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ASSESSMENT OF VASCULAR REACTIVITY
CHANGES IN INSULIN RESISTANCE AND THEIR
ROLE IN BLOOD PRESSURE ELEVATION

JON OWEN CURWEN B.Sc. (Hons)

thesis submitted in fulfilment of the requirements of the
Open University for the degree of Master of Philosophy

October 1997

Zeneca Pharmaceuticals in collaboration with the
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Assessment of vascular reactivity changes in insulin
resistance and their role in blood pressure elevation.

Jon Owen Curwen B.Sc.(Hons)

Submitted for the Degree of Master of Philosophy

1997

Abstract

The aim of this thesis was to investigate the link between changes in vascular reactivity and blood pressure in genetic and induced animal models of insulin resistance. Initially, the suitability of a pithed preparation to study vascular reactivity was established.

Attempts were made to alter the insulin sensitivity and hence the vascular reactivity of a group of AP Wistar rats by placing the animals on a high fat diet. The diet failed to alter the vascular reactivity of the animals tested. Further studies in different rat strains using different diets would be needed to properly investigate the effects of an induced insulin resistant state.

It was found that the insulin resistant obese AP Zucker rat had an increased vascular reactivity compared to the lean, insulin sensitive, AP Zucker rat. It was also found that the obese rat was hypertensive compared to the lean rat, although the difference in blood pressure was slight.

Pioglitazone, an insulin sensitising agent, decreased both blood pressure and vascular reactivity in the obese Zucker rat. However, despite having mild hypotensive effects, pioglitazone had no effect on the vascular reactivity of either the lean AP Zucker rat, the AP Wistar rat or the AP Spontaneously Hypertensive rat.

Two other insulin sensitising agents, vanadyl sulphate and ZD2079 (a β_3 -adrenoceptor agonist) failed to alter the vascular reactivity of either lean or obese AP Zucker rats. This confounded the results obtained when using the other insulin sensitising agent pioglitazone.

Overall, these results indicate a strong association between insulin resistance, vascular reactivity and increased blood pressure but a direct cause and effect relationship has been difficult to demonstrate.

Declaration

I hereby declare that the following thesis is based on the results of investigations conducted by myself and that this thesis is of my own composition. Work other than my own is clearly indicated in the text. None of the work contained in this thesis has been previously presented, in whole or in part, for a higher degree.

Jon Owen Curwen.

Publications

Some of the work presented in this thesis has been published as part of the following papers.

Curwen, J.O., Johnson, I.R. and Hatton, R. (1994)

"Pioglitazone, an orally active anti-diabetic agent, decreases the vascular reactivity of obese Zucker rat but not that of Spontaneously Hypertensive rats".

Brit. J. Pharmacol. **112**: Iss S: U118.

Johnson, I.R., Curwen, J.O., Wilbraham, J.M. and Hatton, R. (1994)

"Differences in blood pressure, heart rate and vascular reactivity in the obese and lean Zucker rat".

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Contents

<u>Section One Introduction</u>	1
1.1 Aims of the project	2
1.2 The definition of insulin resistance	2
1.3 Insulin resistance in disease states	2
1.4 Insulin resistance as a feature of Syndrome X	3
1.5 Mechanisms by which insulin resistance may lead to hypertension	4
1.6 Clinical observations and population studies	9
1.7 Animal models of insulin resistance	16
1.8 Potential drug treatments of insulin resistance	17
1.9 Aims of the project - restatement	19
 <u>Section Two Materials and Methods</u>	 20
2.1 Materials	21
2.2 Animals tested	21
2.3 Methods	22
2.3.1 Measurement of blood pressure and vascular reactivity in conscious and anaesthetised rats	22
2.3.2 Measurements of vascular reactivity in pithed rats	23
2.3.3 Measurements of blood pressure and heart rate in conscious rats by radio-telemetry	24
2.3.4 High fat diet preparation	27
2.3.5 Administration of compounds	27
2.3.6 Statistical methods	28

Section Three Results

3.1 Initial AP Zucker rat experiments	30
3.2 The effect of a high fat diet on the vascular reactivity of the AP Wistar rat	41
3.3 The effects of pioglitazone on the vascular reactivity of the AP Zucker rat	45
3.4 The effects of pioglitazone treatment on the vascular reactivity of the AP Wistar and AP SH strain rats	52
3.5 The effects of pioglitazone on the blood pressure of the AP SH rat as measured by a radio-telemetry system	56
3.6 The effects of two other insulin sensitising agents on the vascular reactivity of the AP Zucker rat	60

<u>Section Four Discussion</u>	69
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<u>Appendix One</u>	83
---------------------	----

<u>References</u>	89
-------------------	----

Section One

Introduction

(1.1) Aims of this Project.

As will be discussed in detail later, it has been postulated in the literature that insulin resistance and associated hyperinsulinaemia have an association with a hypertensive state. Described below are the mechanisms by which insulin (and hence resistance to the action of insulin) may both increase and decrease blood pressure (BP).

The aim of this project/thesis was to attempt to identify possible alterations in the peripheral vasculature of insulin resistant animal models and to assess the physiological significance of any such alterations. In particular it was hoped to determine whether functional differences between the vasculatures of insulin resistant and non insulin resistant animals could be demonstrated and whether these differences were related to blood pressure levels and sensitive to insulin sensitising agents.

(1.2) The Definition of Insulin Resistance

Insulin resistance (IR) is defined as a condition in which a sub-optimal metabolic response is produced by a given amount of insulin. The main impairment of metabolism measured as a marker of IR is a decrease in insulin stimulated glucose uptake by skeletal muscle tissue. The main clinical markers of IR are impaired glucose tolerance and compensatory hyperinsulinaemia.

(1.3) Insulin Resistance in Disease States

Himscliffe (1936) first observed that diabetes mellitus occurred in two forms. Himscliffe observed that some diabetic patients could be maintained by insulin injection and he termed this form of diabetes as type I (since termed insulin dependent diabetes mellitus or IDDM). The author also found that other patients had a form of diabetes which could not be controlled by exogenous insulin and he described this form of the disease as type II (since termed non insulin dependent diabetes mellitus or NIDDM).

IR occurs as a feature of both IDDM and NIDDM diabetes (**DeFronzo et al.**, 1982) but is pathologically most important in NIDDM. Increased hepatic glucose production and impaired insulin secretion exist alongside peripheral IR in NIDDM but it is the IR which is described as the most important contributor to the overall hyperglycaemic state (**Suter et al.**, 1992).

IR also occurs in other disease states such as dyslipidaemia (**Hunt et al.**, 1989), other metabolic conditions such as leprechaunism, and hypertension (**Ferrannini et al.**, 1987). The prevalence of IR and associated hyperinsulinaemia in hypertension will be discussed in more detail later.

(1.4) Insulin Resistance as a feature of "Syndrome X"

Reaven (1988) was the first to link a group of co-existing pathological conditions and describe their association as "Syndrome X". This syndrome describes a condition in which IR, hyperinsulinaemia, impaired glucose tolerance, dyslipidaemia and hypertension are all present. This condition is distinct from cardiological "Syndrome X", described by **Kemp** (1973), which describes a condition in which patients with anginal pain demonstrate electrocardiogram abnormalities on exercise but no angiographic indicators of atherosclerotic coronary artery disease. All following references to Syndrome X refer to the condition as described by Reaven rather than the cardiological condition described by Kemp.

Following Reaven, **Kaplan** (1989) described insulin resistance as the central feature of a group of linked conditions which predispose to cardiovascular disease. He described glucose intolerance, dyslipidaemia, hypertension and obesity as the "deadly quartet".

Table 1 The features of "Syndrome X" (from Reaven 1988)

Resistance to insulin-stimulated glucose uptake
Glucose intolerance
Hyperinsulinaemia
Increased VLDL triglyceride level
Decreased HDL cholesterol level
Hypertension

Possibly the most significant suggestion made by Reaven about Syndrome X is that insulin resistance may not be just a co-existing factor in the syndrome but may exist as a direct causal factor in the development of hypertension and coronary artery disease (especially due to the increased plasma insulin concentration which arises from the insulin resistant state). Experimental evidence for this was put forward.

Reaven described an experiment in which a group of rats were made insulin insensitive by replacing their normal diet with a high fructose diet (i.e., one in which all the carbohydrate was replaced with fructose). These rats went on to develop a degree of hypertension (compared to a group of animals allowed a normal diet) associated with the development of insulin resistance and hyperinsulinaemia. Reaven concluded that in this experiment the high fructose diet caused a degree of insulin resistance which in turn led to a compensatory hyperinsulinaemia. Reaven assumed that this hyperinsulinaemia had a direct causal relationship with the hypertensive change observed.

(1.5) Mechanisms by which Insulin Resistance may lead to Hypertension.

Various mechanisms have been put forward by several authors to explain how IR and associated hyperinsulinaemia may act to produce a hypertensive change. Brief outlines of each of the various possible mechanisms are given below.

(1.5.1) Insulin and central nervous system (CNS) stimulation.

The literature has long suggested that an increased plasma insulin level (as found in insulin resistant states) can be associated with increased plasma catecholamine levels (Rowe et al., 1981 and Christensen et al., 1980). An increased plasma catecholamine level is indicative of an increased level of sympathetic nervous system (SNS) activity. It has been demonstrated that insulin is capable of increasing the activity of the SNS. Stimulation of sympathetic nerves to the cardiovascular system can cause increases in vascular tone and increases in cardiac output (via increases in stroke volume and heart rate). The consequence of this stimulation would be an acute increase in systemic BP.

Exogenous insulin infusion has been shown to increase the levels of sympathetic nervous activity in both animal models (**Tomiyama et al.**, 1992 and **Liang et al.**, 1982) and in humans (**Lembo et al.**, 1992 and **Anderson et al.**, 1991). Insulin may act to increase the activity of the SNS in several ways including alterations in baroreflex function, modulation of neurotransmitter release from peripheral nerve endings or direct stimulation of the higher CNS centres.

Insulin binding has been demonstrated in the following areas of the hypothalamus: the median eminence (**Landau et al.**, 1983), the dorsomedial hypothalamus, the arcuate nucleus and the ventromedial hypothalamus (**Wilcox et al.**, 1989). **Muntzel et al.**, (1995) demonstrated that one area of the hypothalamus in particular was important in the mediating the effects of insulin. This area is the anteroventral third ventricle hypothalamic (AV3V) region. Stimulation of this region resulted in an increase in sympathetic outflow to lumbar regions but not in renal or adrenal nerves. Muntzel and co-workers demonstrated that lesioning of the AV3V region prevented insulin-induced increases in sympathetic nervous output.

Given that insulin may be able to induce BP increases by the above mechanism, it may be concluded that a long lasting hyperinsulinaemic state (present as part of an insulin resistant state) may result in increased sympathetic tone and thus predispose to a hypertensive state. Despite this, however, evidence exists which suggests that acute administration of physiological insulin concentrations to normotensive subjects produces increases in sympathetic outflow without any increase in BP. In fact, acute insulin administration can cause slight falls in BP even when accompanied by increases in heart rate which is an indication of increased sympathetic drive (**Baron et al.**, 1993). This observation would suggest that it may be unlikely that an increased sympathetic outflow driven by hyperinsulinaemia, without any other pathological change, would explain the hypertension seen in insulin resistance.

(1.5.2) Insulin and the Kidney.

The work of Guyton in the 1950's demonstrated that no hypertensive state can be maintained if renal function is normal. This is due to the effect of "pressure natriuresis". Hypertensive changes induce increased natriuresis from normally functioning kidneys (thus leading to reductions in plasma volume) which would cause lowering of systemic BP. According to this principal, for insulin resistance to be a causal factor in a prolonged hypertensive change some renal pathology would have to be present.

Several authors have suggested that insulin can influence events in the kidney. Literature reports have indicated that insulin has activity in the kidney of dogs (**DeFronzo et al.**, 1976) and humans (**DeFronzo et al.**, 1975). These reports state that insulin acts to increase sodium (and hence water) reabsorption in the proximal convoluted tubule.

Endre et al., (1994) demonstrated that patients with a family history of hypertension were insulin insensitive in terms of the promotion of glucose uptake compared to a group of patients without a family history of hypertension (i.e., insulin resistant as per the normal definition of the term). Despite this, both groups of patients demonstrated equal levels of insulin sensitivity in insulin-induced tubular sodium reabsorption in the kidney. This study implies that the hyperinsulinaemia associated with Reaven's Syndrome X may produce chronic sodium retention and hence hypertension as renal insulin sensitivity is retained despite the presence of an overall insulin resistant state.

(1.5.3) Insulin as a Growth Factor

It has been demonstrated in several studies that insulin acts as a growth factor on vascular tissues. A review of these reports has been carried out by **Stout** (1990). A long term consequence of chronic hyperinsulinaemia may be a thickening of resistance vasculature leading to an increase in peripheral resistance. This increase in resistance may promote a hypertensive change.

(1.5.4) Insulin action on vascular reactivity.

Insulin may play a role in the regulation of systemic BP via its ability to induce relaxation in vascular tissue. The importance of changes in the peripheral vasculature in insulin resistant conditions (both clinical and experimental) in the overall hypertensive state associated with the condition have still not been clearly demonstrated.

Some evidence is beginning to emerge demonstrating the importance of peripheral mechanisms in the hypertensive condition of experimental IR models. **Zemel et al.**, (1992) demonstrated that the insulin resistant obese Zucker rat was hypertensive compared to its lean counterparts without CNS contribution. Zemel demonstrated this by treating conscious obese rats with the ganglionic blocking agent Ecolid (chlorisondamine chloride) and demonstrating that they remained hypertensive compared to lean rats treated with the same agent.

In another study, **Zemel et al.**, (1991) demonstrated that aortae from obese rats were hyper-reactive to vasoconstrictor agents *in vitro* compared with tissues taken from lean rats. The authors went on to demonstrate that tissues obtained from the lean animals were sensitive to insulin induced relaxation but tissues from the obese animals were insensitive. **Cox and Kikta** (1992) also demonstrated that aortae from obese Zucker rats demonstrated a greater response to contractile agents compared with tissues obtained from lean animals. When these results are considered alongside the *in vivo* data obtained using ganglionic blockade outlined above, Zemel's work indicates that obese Zucker rats possess differences in their peripheral vasculature compared to their lean counterparts which may predispose them to hypertension.

Other studies have been published which support the results of Zemel and his co-workers. As far back as 1977, **Alexander and Oake** were able to demonstrate that insulin attenuated constrictor responses in the tail artery of insulin sensitive male rats (these results could not be repeated in female animals). In both non-obese dogs and in patients, acute and more chronic hyperinsulinaemia produced reductions in both total peripheral resistance (indicating a vascular relaxation in resistance vessels) and BP. These observations were

made despite increases in sympathetic nervous activity occurring simultaneously (**Hall et al.**, 1992, **Anderson et al.**, 1991 and **Weidmann**, 1991).

In a more direct *ex vivo* study **McNally et al.**, (1995) obtained resistance vessels from healthy volunteers and assessed the sensitivity of these vessels to constrictor agents in the presence and absence of insulin. It was found that noradrenaline-induced contractions of the vessels were significantly attenuated by insulin. Increasing levels of insulin caused dose-related attenuation of noradrenaline without affecting acetylcholine induced relaxation in this study. This study confirms that the attenuation of contractile events produced by insulin in tissues obtained from rats as seen by Zemmel and co-workers may occur in humans too.

Conversely, it has been demonstrated that insulin fails to cause any decrease in the peripheral vascular resistance in obese dogs (**Hall et al.**, 1992). Patients with essential hypertension and NIDDM have both been shown to have an increased responsiveness to vasoconstrictor agents (**Ferrari and Weidmann**, 1991).

From the studies outlined above, it may be deduced that insulin has a potential physiological relaxant role in the peripheral vasculature. It may be expected then, in normal animals and man, that the hyperinsulinaemia present as part of an insulin resistant state should produce a hypotension rather than the hypertension which is actually seen. Evidence from *in vitro* testing of tissues from insulin resistant models and *in vivo* examinations of insulin resistant models and patients suggests, however, that they are insensitive to this relaxant effect of insulin. If insulin does indeed play a role in the normal control of vascular tone, resistance to this relaxant modulation may contribute to a hypertensive change.

The mechanisms by which pathological hyperinsulinaemia or insulin resistance may modulate blood pressure, as discussed in this section, are summarised in table two (on page 19a).

(1.6) Clinical observations and Population Studies

1.6.1 Clinical studies of hypertensive patients

The first study of the role of insulin and BP regulation was published in 1966 in which **Welborn et al.**, demonstrated that a group of 19 hypertensive subjects (who had diastolic BP above 110 mmHg) had elevated plasma insulin levels compared to a group of 45 normotensive subjects. It was found that the relationship between plasma insulin and diastolic BP was present in the hypertensive group even when the factors of age and anti-hypertensive therapy were taken into account. Interest in the potential link between plasma insulin levels (as well as IR and its associated features) and BP levels grew in the mid 1980's following Reaven's suggestion that IR, and hence hyperinsulinaemia, may play a causal role in the development of hypertension.

Ferrannini et al., in 1987, published a study carried out in a small group of subjects, 7 males and 6 females, with moderate to severe untreated hypertension. These hypertensive subjects were aged 38 ± 2 years and had a body mass index (BMI) of 26 ± 1 (i.e., moderately overweight). The subjects BP levels were 165 ± 5 mmHg (systolic), 132 ± 4 mmHg (mean) and 112 ± 3 mmHg (diastolic). When compared to a normotensive control group (of 7 males and 4 females), the hypertensive subjects demonstrated a significantly lower insulin-stimulated glucose uptake. During an insulin infusion to maintain a steady plasma insulin concentration ($60 \mu\text{U ml}^{-1}$), insulin mediated glucose disposal (IMGD) was $3.80 \pm 0.32 \text{ mg kg}^{-1} \text{ min}^{-1}$ in the hypertensive group and $6.31 \pm 0.42 \text{ mg kg}^{-1} \text{ min}^{-1}$ in the control group ($P < 0.001$). It was also found that glucose uptake was inversely related to systolic BP and mean BP ($r = 0.76$ for both, $P < 0.001$).

In a slightly larger study, **Pollare et al.**, (1990) studied the plasma insulin, plasma glucose and rate of IMGD in a group of 143 newly diagnosed hypertensive subjects and a control group of 51 normotensive subjects. The hypertensive subjects were divided into non-obese and obese groups using a body mass index (BMI) of 27 for male subjects and 26 for females as cut-off points. The authors found that IMGD was significantly decreased in both the obese hypertensives ($5.1 \pm 2.1 \text{ mg kg}^{-1} \text{ min}^{-1}$, $P < 0.05$) and the non-obese hypertensives

($7.2 \pm 2.1 \text{ mg kg}^{-1} \text{ min}^{-1}$, $P < 0.05$) when compared to the normotensive group ($8.4 \pm 1.8 \text{ mg kg}^{-1} \text{ min}^{-1}$). The authors also found that after controlling for sex, age and BMI the hypertensive subjects had a higher fasting plasma insulin than the normotensive subjects ($P < 0.05$).

The three studies outlined above demonstrated that relatively small groups of hypertensive patients (both obese and non-obese) had some of the features of IR. **Lind, Berne and Lithell**, (1995) published a study in which the prevalence of IR in a large group of hypertensive subjects was evaluated. The hypertensive subject group was composed of 420 patients with diastolic BP of over 95 mmHg either never treated with anti-hypertensive therapy or placed on placebo for 4 weeks prior to the study. The hypertensive group were also selected on the basis of a fasting blood glucose level of $> 6.7 \text{ mmol l}^{-1}$. A "healthy" control group was also tested. This control group consisted of 51 subjects with a diastolic BP below 95 mmHg and fasting blood glucose of $< 6.7 \text{ mmol l}^{-1}$. Using a value for IMGD of $4.4 \text{ mg kg}^{-1} \text{ min}^{-1}$ as a cut-off (as accepted in most of the literature), it was found that 27 % of the hypertensive group were insulin resistant. The authors found that the mean IMGD value in the control group tested was $8.2 \pm 1.9 \text{ mg kg}^{-1} \text{ min}^{-1}$. The authors also suggested that the results from eight different studies in their laboratory on subjects with normal insulin sensitivity indicated that a IMGD value of $5.5 \text{ mg kg}^{-1} \text{ min}^{-1}$ was a more accurate cut-off level to use to define clinical insulin resistance. When applying this $5.5 \text{ mg kg}^{-1} \text{ min}^{-1}$ value, 43 % of the hypertensive subjects tested in this study were found to be insulin resistant.

The figure of 43 % quoted by Lind et al., above is similar to that quoted by **Zavaroni et al.**, (1991) in a study of the incidence of hyperinsulinaemia in another group of patients with essential hypertension. This study involved 41 hypertensive subjects and a normotensive group of 41 age matched subjects. It was found that 90 % of the normotensive subjects had a plasma insulin level of $< 80 \text{ } \mu\text{U ml}^{-1}$ 2 hours following an oral glucose load. Using the figure of $80 \text{ } \mu\text{U ml}^{-1}$ as a cut-off it was found that 41 % of the hypertensive subjects were hyperinsulinaemic.

1.6.2 Epidemiological studies of insulinaemic/insulin resistant patients

The studies described above in section 1.5.1 indicate that IR and associated hyperinsulinaemia are features of at least a percentage (although by no means all) of subjects with essential hypertension. Despite this evidence a link between insulin sensitivity and associated hyperinsulinaemia with BP levels has been difficult to demonstrate in population studies. In the early studies of this relationship investigators examined the assumption that insulinaemia alone was the causal factor in BP elevation (due to increased CNS output and decreased renal output as described in section 1.4 above). Due to this assumption most of the early studies of IR and BP concentrated purely on the potential link between plasma insulin levels and BP levels.

One of the most widely quoted papers in which a positive correlation between plasma insulin levels and BP was demonstrated was that published by **Modan et al.**, (1985). This study was part of a larger study entitled the "Israel study of Glucose intolerance, Obesity and Hypertension". A representative sample of 2,769 subjects was selected from the original sample of 5,711 subjects. After accounting for the removal of refusals, known diabetics and technical problems 2,475 subjects underwent a full oral glucose tolerance test. Within this sample it was found that hypertension and glucose intolerance showed a highly significant association ($P < 0.0001$) which was independent of sex, age, obesity and anti-hypertensive medication. Although significant, the relationship between insulin level and hypertension was less strong than that of obesity to hypertension which, in turn, was less strong than that of glucose intolerance to hypertension. The strongest correlation with hypertension was a condition in which hyperinsulinaemia, obesity and glucose intolerance were all present.

The type of results obtained by Modan et. al., have also been demonstrated by **Lucas et al.**, (1985) in a group of 33 "very obese" women. Lucas and co-workers found that both systolic and diastolic BP were significantly related to fasting plasma insulin levels even when age, weight and serum glucose levels were taken into account. It must be noted that in this study the relationship between insulin and BP was strongest in subjects with a family history of hypertension. In another study of obese subjects (10 women and 25 men),

Manicardi et al., (1986) found a significant correlation between 2 hour post load insulin levels and BP in hypertensive but not normotensive subjects. **Rose et al.**, (1986) made a study of 19 Vietnam veterans with leg amputation just below the knee and a comparison group of Veterans with an arm amputation. The important difference between the two groups was the fact that the subjects with an arm amputation were more mobile than the subjects with a leg amputation who could exercise less. It was found that the subjects with a leg amputation had a greater BMI than the comparator group. The authors found that both insulin level following an oral glucose load and body fat content were strongly and independently correlated to diastolic BP.

The studies outlined above were mostly composed of obese white subjects. It is obvious from the literature that the relationship between insulin and BP is much less consistent across other ethnic groups. In a study of Pima Indians (native Americans from the Gila river Indian community in Phoenix, Arizona) carried out by **Saad et al.**, (1990), it was found that 2033 subjects, who were taking no anti-hypertensive or diabetic therapy, demonstrated no correlation between their fasting plasma insulin levels and their BP. In a larger population, which was not selected on the basis of therapy taken, it was found that hypertension was positively correlated with age, male sex, BMI, glucose tolerance and fasting insulin levels (but not 2 hour post load insulin levels). The authors suggest that the antihypertensive therapies taken by some subjects were affecting plasma insulin levels and may have altered the insulin and BP relationship in this larger population.

In 1991 **Saad et al.**, again demonstrated that no correlation existed between plasma insulin levels and BP in a group of 116 Pima Indians. In this new study a group of white (n=53) and a group of black (n=42) North Americans were also tested. It was found that after accounting for age, sex and BMI, mean BP was significantly correlated to fasting insulin levels ($P<0.01$) in the white population alone.

Cruickshank et al., (1991) also described an ethnically mixed study consisting of 106 Afro-Caribbean, 107 Gujerati Indian and 101 white European subjects. In this study no correlation between any insulin measure and BP could be found in any of the groups.

Cambien et al., (1987) and **Muller et al.**, (1993) described different studies of white

subjects in which a strong correlation between hypertension and an elevation of BMI, plasma glucose and plasma insulin was found but no correlation between insulin levels and BP was found when the other factors were taken into account.

1.6.3 Blood pressure levels in two other hyperinsulinaemic conditions

Most of the earlier literature studies of IR outlined above assume that the compensatory hyperinsulinaemia resulting from the insulin resistant state to be the potential driving force behind a hypertensive change via increased CNS output, decreased renal function or both (these mechanisms were described in sections 1.4.1 and 1.4.2 above). Several studies involving BP measurement have been carried out in patients with conditions other than IR in which a hyperinsulinaemic state is present.

Vettor et al., (1994) describe a study in 37 patients with insulinoma (insulin secreting tumours) admitted to the Institute Semeiotica Medica in Padua Italy between 1966 and 1990. These patients were 21 females and 16 males of average age 47.1 ± 2.8 years with a mean average of symptoms of 33.1 ± 5.1 months. A control group of patients, without insulinoma, of equivalent age and BMI to the insulinoma bearing patients were also examined. It was found that the insulinoma patients had much higher plasma insulin levels than the control subjects (407.2 ± 96 pmol l⁻¹ against 42.0 ± 4.2 pmol l⁻¹) but had no significant differences in either systolic BP (135 ± 3 mmHg against 132 ± 8 mmHg) or diastolic BP (87 ± 1 mmHg against 86 ± 1 mmHg). However it must be noted that the BP of the patients fell slightly following removal of the insulinoma. This BP fall was small but statistically significant (the diastolic BP fell from 87 ± 1 mmHg with the tumour present to 80 ± 2 mmHg after its removal, $P < 0.05$).

Tsutsu et al., (1990) also studied the BP levels of a small number of patients with insulinomas. The authors found that 7 patients with a mean plasma insulin level of 568 pmol l⁻¹ prior to the removal of their tumour had a mean systolic BP of 127 ± 15 mmHg and a mean diastolic pressure of 74 ± 10 mmHg (i.e., below the level of clinical hypertension). After the removal of the tumour the patients systolic pressure fell slightly to 120 ± 14 mmHg but no correlation existed between BP fall and the fall in plasma insulin

levels. **Pontiroli et al.**, (1992) described a study of 13 patients with insulinoma and 6 with "non-tumour hypoglycaemia and hyperinsulinaemia". It was found that 3 out of the 19 patients were hypertensive and that this figure fell to one patient when those patients with a history of familial hypertension were excluded. **Sawicki et al.**, (1991) published a comparative study of 34 patients with insulinoma and 34 age and sex matched controls. It was found that both groups had very similar BP levels (131/81 mmHg in the insulinoma patients and 130/80 mmHg in the control group).

A much larger retrospective study of the BP levels of patients with insulinomas was described by **O'Brien et al.**, (1991). The BP levels of 250 patients admitted to the Mayo Clinic between 1927-1991 were measured. Nine patients were excluded on the grounds that they were being treated for pre-existing hypertension. In the remaining group of 241 patients, who had a median age of 41 years and a median duration of symptoms of 1.9 years, it was found that 31 patients (12 %) had clinical hypertension (i.e., a BP level greater than 145/90 mmHg). This figure did not exceed that expected from a representative sample of the general population.

Polycystic ovary syndrome (PCO) is a disorder of women characterised by chronic anovulation and hyperandrogenism. The condition is associated with insulin resistance, hyperinsulinaemia and an increase in android obesity. **Chang et al.**, (1983) demonstrated that IR and hyperinsulinaemia were present in lean patients with PCO indicating that PCO is an insulin resistant state independent of obesity. In 1992 **Zimmerman et al.**, published a study of 14 subjects with PCO (10 obese and 4 non-obese) alongside a control group of insulin sensitive subjects without PCO. It was found that the two groups had very similar BP levels, the PCO group having mean blood pressure values of 121/76 mmHg and the control group having mean blood pressure values of 118/73mmHg.

1.6.4 Insulin Resistance not hyperinsulinaemia related to Blood Pressure

Saad et al., (1994) demonstrated, in a group of 183 non-diabetic subjects, that hypertensive and normotensive subjects did not have significantly different fasting plasma insulin levels. Despite this the authors found a significantly higher 2 hour insulin level

following an oral glucose load in the hypertensive subjects compared to the normotensive subjects. From this observation the authors concluded that IR itself had a greater association with BP than compensatory hyperinsulinaemia. This observation fits with the data obtained from studies in groups of hypertensive subjects by both **Ferraninni et al.**, and **Pollare et al.**, (as described above in section 1.6.1 above). These studies linked direct measurements of insulin sensitivity (such as insulin mediated glucose disposal) with BP levels and demonstrated a positive correlation between the two.

Despite the fact that measurements of insulin sensitivity appear to be more closely related to BP levels, and to hypertension in particular, it must be added that the ethnic differences seen in studies of pure hyperinsulinaemia are still present in the studies involving insulin sensitivity. Studies have demonstrated that Pima Indians are insulin resistant as well as hyperinsulinaemic but not hypertensive (**Saad et al.**, 1991). As described previously, another condition in which IR is present is polycystic ovary syndrome. **Zimmerman et al.**, (1992) failed to find evidence of BP changes in 14 patients with polycystic ovary syndrome despite profound IR.

Summary

The literature evidence described above (in all of section 1.6) points to the fact that at least a proportion of essential hypertensives demonstrate some of the features of IR, especially hyperinsulinaemia. It is also clear that a condition in which insulin resistance, hyperinsulinaemia and android obesity are all present (i.e., the various components of Syndrome X) is strongly correlated with an increased BP level. The link between pure hyperinsulinaemia and BP is at best controversial (i.e., when other factors, especially obesity, are corrected for). Insulinaemic conditions, such as polycystic ovary syndrome and insulin secreting tumours, in themselves are not linked with increases in BP. More recently a view is emerging that insulin sensitivity (and hence insulin resistance) rather than hyperinsulinaemia has a stronger link with BP levels but again, due to factors such as ethnic differences, the evidence for this is not conclusive when other factors are corrected for.

A potential weakness in the literature on insulin resistance and its link with BP levels, especially in the population studies described above, is that many authors have concentrated on attempting to demonstrate a link between a single facet of Syndrome X (especially hyperinsulinaemia) with hypertension. In doing this the authors have selected groups of subjects who do not demonstrate all the features of Syndrome X (obesity tends to be corrected for especially). As insulin resistance may be the causal factor behind these other conditions, the authors risk selecting a non-typical population of insulin resistant subjects to study.

(1.7) Animal Models of Insulin Resistance.

Animal models of IR can be divided into two main categories: induced models (in which IR is induced mostly by dietary alteration) and spontaneous (i.e., genetic) models.

1.7.1 Dietary (Induced) Models.

Several studies have linked alterations in diet with decreases in insulin sensitivity and increases in vascular reactivity. Alterations in diet that have been shown to be effective in the induction of a hypertensive change include increases in the sugar content. One of the earliest of these studies was that carried out by Reaven and co-workers which was outlined in section 1.3 above. **Martinez et al.**, (1994) also demonstrated that a high fructose diet induced insulin resistance, hyperinsulinaemia and a rise in BP in normal mongrel dogs rather than in rats. **Zein et al.**, (1990) demonstrated that a diet high in sucrose could further elevate the BP of the already hypertensive SH rat.

Other workers have demonstrated that IR may be induced by increases in dietary fat composition. **Pedersen et al.**, (1991) produced IR and a decrease in the expression of GLUT-4 (a glucose transporter) by imposing a high fat diet on a group of rats. **Storlien et al.**, (1991) also induced an insulin resistant state in rats by means of a high saturated fat diet.

1.7.2. Spontaneous Models.

The two main animal models of IR are the ob/ob mouse and the obese Zucker rat. The Zucker rat is a spontaneously obese animal and was first described by **Zucker and Zucker** in 1967. The obese Zucker rat has two distinct two phenotypes, lean and obese. The obese rat inherits its condition due to an autosomal recessive trait (**Bray 1977**). The obese rat has been described as a potential model of both obesity and of hypertension as the obese rat demonstrates a higher BP than its lean littermate (**Boese et al., 1986** and **Kasiske et al., 1985**). The obese Zucker rat has been described by **Kurtz et al., (1989)** as insulin resistant (and hypertensive) compared to lean littermates.

The ob/ob mouse is also a spontaneously obese animal model with a degree of insulin resistance. The ob/ob mouse possess genetically transmitted obesity, hyperglycaemia and hyperinsulinaemia (**Flatt and Bailey, 1981**).

The spontaneously hypertensive (SH) rat has also been described as another potential spontaneous model of IR. The SH rat has long been recognised as a model of hypertension and also of increased vascular reactivity (**Field et al., 1972**). Current literature reports are divided as to whether the SH rat may be described as an IR model eg., **Hulman et al., (1993)** found the SH rat to be insulin resistant while **Buchanan et al., (1992)** failed to demonstrate IR in the strain.

(1.8) Potential Drug Treatments of Insulin Resistance.

Literature reports have described a number of chemical agents which increase insulin sensitivity and are therefore of use in reversing insulin resistance in animal models. Agonists of a recently described β -adrenoceptor agonist subtype (the β_3 -adrenoceptor) have been shown to be effective in increasing insulin sensitivity in animal models. **Cawthorne et al., (1992)** described the effects of the selective β_3 -adrenoceptor agonist BRL 35135 in the obese Zucker rat. In this study it was found that BRL 35135 normalised the glycaemic state of the obese rat at a dose of $23.4 \mu\text{g kg}^{-1} \text{ day}^{-1}$. The authors also

demonstrated that BRL 35135 was effective clinically in improving glucose tolerance when dosed to obese subjects. **Connacher et al.**, (1992) also demonstrated that another β_3 -adrenoceptor agonist, BRL 26830A, was effective in reducing plasma insulin in obese subjects. **Wilbraham et al.**, (1994) demonstrated that ZD2079 was capable of improving glucose tolerance and decreasing plasma glucose and insulin when dosed to insulin resistant, ob/ob mice.

Vanadium containing compounds have been described in the literature as being of use in IR animal models. **Lonnroth et al.**, (1993) suggested that vanadate acts in an insulin-like manner in human adipocytes (i.e., that vanadate acts as an insulin mimetic in these cells). This observation was supported by **Wallburg-Henriksson et al.**, (1993) who demonstrated that vanadate was capable of increasing glucose oxidation and synthesis in the skeletal muscle of obese subjects. **Brichard et al.**, (1990) described improvements in glucose homeostasis in the insulin resistant ob/ob mouse as a consequence of sodium orthovanadate administration. More directly relevant in the context of the peripheral vascular defects of insulin resistant animal models are the observations of **Ozcelikay et al.**, (1994). These authors described a normalisation of the increased vascular responsiveness of aortae from streptozocin (STZ) treated diabetic rats following vanadyl sulphate dosing.

One of the most interesting and most studied class of compounds which may be of use in insulin resistant states is the thiazolidenediones. This class of agent includes pioglitazone, ciglitazone and CS-045. These agents have been shown to be true insulin sensitising agents in that they require the presence of insulin to exert a physiological effect (**Hofmann and Colca** 1992).

CS-045 has been shown to improve insulin sensitivity and lipid disorders. **Fujiwara et al.**, (1991) demonstrated that chronic CS-045 treatment in diabetic mice (db/db strain) produced reductions in hyperglycaemia, glucose intolerance, plasma triglycerides and free fatty acid levels. Despite these results, CS-045 has been shown to be ineffective in another diabetic model, the STZ treated mouse (**Fujiwara et al.**, 1988). The agent has also been shown to be effective in patients as well as in animal models. **Iwamoto et al.**, (1991) demonstrated that CS-045 treatment of patients with NIDDM produced an improvement in glucose tolerance without a stimulation of insulin secretion (which suggests that an

increase in insulin sensitivity had occurred). These results were supported by **Suter et al.**, (1992) who also demonstrated improvements in glucose tolerance, plasma insulin levels and plasma free-fatty acid levels in CS-045 treated NIDDM patients.

Pioglitazone, another thiazolidenedione agent, has also been shown to be useful in reducing the level of IR in animal models (**Ikeda et al.**, 1990 and **Sugiyama et al.**, 1990). Pioglitazone has also been shown to have effects on BP in animal models of IR. **Kemnitz et al.**, (1994) demonstrated that pioglitazone treatment of obese insulin resistant Rhesus monkeys produced reductions in plasma insulin and glucose and a significant fall in mean arterial BP.

(1.9) Aims of this Project - Restatement.

As outlined in the introduction above, it has been postulated in the literature that IR and associated hyperinsulinaemia have an association with a hypertensive state. Also described above are the mechanisms by which insulin (and hence resistance to the action of insulin) may both increase and decrease BP.

The aim of this project/thesis was to attempt to identify possible alterations in the peripheral vasculature of insulin resistant animal models and to assess the physiological significance of any such alterations. In particular it was hoped to determine whether functional differences between the vasculatures of insulin resistant and non insulin resistant animals could be demonstrated and whether these differences were related to BP levels and sensitive to insulin sensitising agents.

Table Two The potential mechanisms by which insulin, and hence resistance to the actions of insulin, can alter blood pressure and in turn induce a hypertensive state.

<u>Target cell or organ</u>	<u>Insulin Effect</u>	<u>Mechanism of Blood Pressure Change</u>	<u>Hypertension due to</u>
Central Nervous System	Increased Sympathetic output	Increased cardiac output and peripheral vascular resistance	Hyperinsulinaemia
Kidneys	Increased Sodium Re-absorption	Increased circulating plasma fluid volume	Hyperinsulinaemia
Vascular smooth muscle cells	Increased mitogenesis	Increased peripheral vascular resistance	Hyperinsulinaemia
Vascular smooth muscle cells	Increased calcium-ATPase pump activity	Decreased peripheral vascular resistance	Insulin resistance
Vascular smooth muscle cells	Decreased L-channel calcium entry	Decreased peripheral vascular resistance	Insulin resistance

Section Two

Materials and Methods

(2.1) Materials

"Intraval" anaesthetic (thiopentone sodium 2.5%, May & Baker)

"Saffan" anaesthetic (steroid mixture consisting of alphaxalone and alphadolene acetate with 20% w/v polyoxyethylated castor oil, Pitman-Moore)

"Fluothane" inhalation anaesthetic (Halothane, Zeneca Pharmaceuticals)

Heparin sodium ("Multiparin", CP Pharmaceuticals) - 5000 units ml⁻¹

Phenylephrine (L-phenylephrine hydrochloride, Sigma)

Angiotensin II (Sigma)

Pioglitazone (Zeneca Pharmaceuticals)

ZD2079 (Zeneca Pharmaceuticals)

Vanadyl sulphate (vanadyl sulphate trihydrate, Aldrich)

(2.2) Animals Tested

Alderley Park Wistar strain rat (AP Wistar rat)

Alderley Park Spontaneously Hypertensive strain rat (AP SH rat)

Alderley Park (AP) lean Zucker rat

Alderley Park (AP) obese Zucker rat

(2.3) Methods

(2.3.1) Measurement of Blood Pressure and Vascular Reactivity in Conscious and Anaesthetised Rats

Male lean and obese Zucker rats were anaesthetised with Saffan (10 mg kg^{-1}) via the tail vein. A small incision was made in the throat of the animal and the right jugular vein and right carotid artery were exposed. The two blood vessels were cannulated with sealed cannulae containing heparinised saline. The arterial cannula consisted of a piece of polythene tubing with an external diameter of 1.27 mm and an internal diameter of 0.86 mm (Portex code 800/100/260/100). The venous cannula was a piece of polythene tubing with an external diameter of 0.8 mm and an internal diameter of 0.4 mm (Portex code 800/100/140/100). The two cannulae were sealed with a pin to prevent both blood loss from the animal and blood leakage into the cannula which may have caused clotting and hence blockage.

A trochar was used to punch a hole in the nape of the animals neck and left in place. The two cannulae were then exteriorised using the trochar. The incision in the throat of the animal was closed using veterinary autoclips.

On the day following surgery, the rats were placed in restraining tubes. The carotid cannula was clamped with a pair of Spencer-Wells artery clamps to prevent blood loss from the rat and the pin removed. The now open end of the cannula was then connected to a heparinised saline filled pressure transducer to enable blood pressure and heart rate recordings to be made. The blood pressure and heart rate recordings were made via a Lectromed MT8P amplifier unit and Astro Med MT95000 16 channel chart recorder.

The animals were allowed to settle in the restraining tubes until steady blood pressure and heart rate values were obtained (typically 20 to 25 minutes). Following this, increasing doses of pressor agent (either phenylephrine or angiotensin II) were administered via the jugular vein cannula. The maximal rise in diastolic pressure above baseline following the administration of the pressor agent was measured.

The typical BP response to phenylephrine was biphasic in nature. A very rapid rise in BP was followed by a longer lasting plateau phase. The diastolic pressure measurement taken was at the secondary, more sustained, phase of the response as this part of the response would be less likely to be affected by speed of administration. The BP of the animal was allowed to return to the original baseline before the next pressor response was induced.

(2.3.2) Measurement of Vascular Reactivity in Pithed Rats.

Obese and lean Zucker rats of either sex were anaesthetised with Saffan (10 mg kg^{-1}) via the tail vein. The trachea of the rat was exposed and cannulated to enable artificial respiration. The rat was then pithed with a metal pithing rod via the eye-orbit and then immediately attached to a small rodent ventilator (Searle Instruments, model 5119). The ventilator supplied a 40 % oxygen and 60 % nitrogen mixture at a volume of 4 ml and at a rate of $55 \text{ strokes min}^{-1}$.

The right carotid artery of the animal was cannulated to enable BP recordings to be made. The arterial cannula consisted of a piece of polythene tubing with an external diameter of 1.27 mm and an internal diameter of 0.86 mm (Portex code 800/100/260/100). The right jugular vein was cannulated to enable drug administration to occur. The venous cannula was a piece of polythene tubing with an external diameter of 0.8 mm and an internal diameter of 0.4 mm (Portex code 800/100/140/100). The body temperature of the animal was maintained at 38°C with a heated blanket. The temperature of the heated blanket was regulated via a thermostat connected to a rectal thermometer (Bioscience CFP 8185).

The carotid artery cannula (filled with heparinised saline) was connected in turn to a saline filled pressure transducer (Bell & Howell 4-422-001). This transducer was in turn connected to a combined amplifier and chart recorder (Lectromed Multitrace 2) which was used to record BP measurements. The BP signal was differentiated to obtain a heart rate measurement which was also recorded.

After surgery was completed, the animal was allowed to equilibrate until steady baseline BP and heart rate values were obtained (this typically took around 20 to 25 minutes).

Following this, increasing doses of pressor agent (either phenylephrine or angiotensin II) were administered via the jugular vein cannula. The maximal rise in diastolic pressure above baseline following the administration of the pressor agent was measured.

(2.3.3) Measurements of Blood Pressure and Heart Rate in Conscious Rats by Radio-Telemetry

The Data Sciences radio-telemetry equipment provides a means of measuring the BP, heart rate and activity of a conscious unrestrained rat remotely. The measurements obtained using this system are free from the stress induced by surgery and restraint.

The Data Sciences radio-telemetry system comprises of a pressure transducer (TA11PA-C40) implanted in the abdomen of a rat which transmits a radio signal indicating the pressure in the aorta of the animal. The implant contains a magnetic switch which can be used to turn the unit off and on (thus prolonging battery life). The signal is sent to a receiver (RA 1010) placed under the plastic cage of the animal and evaluated and recorded automatically by prewritten computer software (Data Quest IV installed on an AST 486 PC).

The system measures absolute pressure within the animal. Atmospheric pressure is measured within the animal housing area (by a pressure reference unit, C11PR) and is subtracted from the pressure recorded within the animal. This correction thus cancels out barometric changes over the course of an experiment and prevents drift in the calibration of the implanted transducer.

The upper and lower pressure readings obtained by the system indicate the systolic and diastolic blood pressures of the animal and the number of pressure peaks per unit time indicate the heart rate of the animal. Alterations in the signal strength detected at the receiver are used to give an indication of animal movement and are stored by the computer as an activity score.

Rats were anaesthetised with "Fluothane" inhalation anaesthetic. The abdomen of the rat was then shaved and the skin of the shaved area was coated with a topical disinfectant. An incision was made with a scalpel on the outer skin to expose the abdominal muscle wall. The muscle wall was then cut along the mid-line and opened. The viscera of the animal was held back with retractors and the abdominal aorta located. The aorta was cleared of connective tissue over a 2-3 cm length and carefully separated from the associated vena cava.

A tie was placed loosely under the aorta. The tie was then lifted to occlude the aorta. A puncture was made in the vessel with a 21 gauge needle (Micro Lance, Becton Dickson) the tip of which had been bent to approximately 90 degrees to the needle shaft. Using the bevel of the needle (held in place in the vessel), the tip of a Data Sciences rodent implant was carefully inserted into the puncture in the blood vessel. Once the tip of the implant catheter had been pushed further into the aorta the needle was removed. A small drop of surgical glue (Vet Bond, 3M) was run down the exposed catheter length to form a seal on the interface between the catheter and the blood vessel. Care was taken to avoid the fixing of the cannula tip into the aorta around the area where the renal arteries branch from the larger vessel. This was done to avoid the possibility of either the cannula, or the glue used to fix it into place, causing a constriction of the renal vessels. Physiological saline was run across the base of the catheter to harden and fix the glue.

A small piece of sterile paper was then placed across the aorta on top of the inserted cannula and glued into place. This paper acted to fix the aorta around the area of the catheter insert to prevent movement which may have lead to the catheter pulling on the aorta (threatening the integrity of the blood vessel).

The implant body was coated in a fine mesh which formed a lip on one side of the implant. This lip was used to stitch the implant onto the inside wall of the abdominal cavity to prevent movement of the implant within the abdomen (again to reduce the possibility of movement of the implant body which may place strain on the blood vessel). The abdominal

muscles were then closed with a single continuous stitch. The ends of the abdominal stitching were trimmed and the skin of the animal closed with surgical autoclips.

Following surgery, the rat was placed in a plastic based cage containing sawdust. This type of animal housing was used for two reasons. If placed in a normal metal cage the rat could cause removal of the autoclips holding the recovering abdominal skin by contact with the metal bars. A metal cage would also interfere with the signal from the implant. Seven days after surgery the autoclips were removed.

Once the autoclips were removed from the animal, the implanted transducer was turned on with a magnet. The animals activity pattern was observed over several days to ensure normal activity rhythms were occurring (i.e., that the animal was active during the night cycle and dormant during the day cycle). Typically the normal activity pattern returned within seven days. The presence of this normal activity pattern indicated that the animal was fully recovered from the stress of surgery and autoclip removal.

Study Protocol

The telemetry study carried out for this project used male AP SH rats aged approximately 3 months at the time of surgery and implantation. Implantation was impossible on younger animals due to the size of the implant body. Once the autoclips were removed after surgery and the animals had fully recovered, the animals were orally dosed once daily with water to accustom them to the dosing procedure. This acclimatisation period lasted for approximately five days.

Once oral dosing failed to produce stress responses in the animals and steady haemodynamic measurements were observed the rats were dosed with polysorbate (i.e., drug vehicle). Polysorbate was dosed once daily for seven days. At the end of this control period pioglitazone was dosed at 30 mg kg⁻¹ to each animal once daily for a further period of seven days.

(2.3.4) High Fat Diet Preparation

The high fat diet was composed of 60 % ground rat diet (Scientific Services), 10 % caesin (Sigma) and 30 % lard (Co-op). A control group of rats was fed ground rat diet only (i.e., the same ground diet as in the high fat diet but without the supplementary caesin and lard).

Each kilogram of the high fat diet was prepared in the following manner. 600 g of ground diet was mixed with 100 g of caesin in a blender. Once these two powders were thoroughly mixed, 300 g of lard was added to the mixture. The lard was added and blended into the mixture in four to five portions rather than a single block to enable efficient mixing. The diet was either placed directly into bowls and placed in the animal housing or stored in a refrigerator to prevent spoilage.

(2.3.5) Administration of Compounds

Pressor agents (phenylephrine & angiotensin II)

Both pressor agents used in the experiments described were administered by the i.v. route and so were dissolved in physiological saline. Both pressor agents were stored in a freezer overnight and on ice during experiments to prevent decomposition.

Pioglitazone

Typically experiments carried out involving pioglitazone consisted of two groups of animals on test, a pioglitazone treated group and a vehicle treated group. Pioglitazone was dissolved in polysorbate vehicle to give a final concentration of 30 mg ml⁻¹. Pioglitazone was dosed orally to provide 30 mg kg⁻¹ to each animal on test in the treated group (i.e., 0.3 ml of the 30 mg ml⁻¹ drug solution was dosed to a 300g animal). Animals in the control group were orally dosed with an equivalent volume of polysorbate (i.e., a 300 g rat was dosed with 3 ml of polysorbate). Dosing occurred once daily (at around 9:00 am) for seven days. After the final dose the animals were pithed and their vascular reactivity to pressor agents was assessed (using the protocol described in section 2.2.1).

(2.3.6) Statistical Methods

All statistical calculations included in this thesis were carried out using the statistic tools of the Microsoft Excel 5 spreadsheet package running on an IBM PS2 PC under the Microsoft Windows 3.11 operating system. The tests used were the paired and unpaired student's t-test. The following statistical convention was used in this thesis :

- * $P < 0.05$
- ** $P < 0.01$
- *** $P < 0.001$

Section Three

Results

3.1 Initial Zucker Rat Experiments

As described in the introduction section, literature evidence suggests that IR may be linked to increased vascular reactivity and hypertension. The following experiments were carried out using small groups of insulin sensitive lean and insulin resistant obese Alderley Park strain Zucker rats. These experiments were carried out as "sighting" experiments both to make an initial assessment of the BP levels and vascular reactivities of the two phenotypes but more importantly to determine the best experimental methodologies and conditions to adopt in studying both phenotypes of the Zucker rat.

3.1.1 Measurement of Blood Pressure and Vascular Reactivity in Conscious Zucker rats

Introduction

The first experiment carried out was designed to provide an insight into the relative BP levels of the lean and obese Zucker rat and also to investigate whether the two phenotypes had different vascular reactivities to i.v. phenylephrine administration.

Five obese AP Zucker rats were used in this study along with five of their lean counterparts. The rats were male and aged three and a half to four months. Briefly, rats were cannulated under anaesthesia and allowed to recover. Measurements of BP and vascular reactivity were made 24 hours later. A full description of the methodology used in this experiment was given in the methods section (in section 2.3.1) but, briefly, basal BP and changes in BP caused by phenylephrine were measured by connecting the arterial cannula of each animal to a pressure transducer while the animals were held in restraining tubes. The pressor agent used in this experiment was phenylephrine at doses of 1, 3, 10 and 30 $\mu\text{g kg}^{-1}$.

Results

Table 3.1 Conscious Blood Pressure values (mmHg) of Lean and Obese Zucker Rats

Obese Rats

	1	2	3	4	<u>Mean</u>	<u>s.e.</u>
Systolic BP	135	135	145	130	136	3.1
Diastolic BP	95	90	105	85	94	4.3

Lean Rats

	1	2	3	4	<u>Mean</u>	<u>s.e.</u>
Systolic BP	150	145	145	135	144	3.1
Diastolic BP	115	115	105	105	110	2.9

$P<0.05^*$

* by unpaired t-test

It was found that the lean Zucker rats had greater BP values than the obese animals (see table 3.1 above). A significant difference was found between the diastolic BP values of the two phenotypes ($P<0.05$ by unpaired t-test) despite the small group sizes tested. However, despite demonstrating a lower basal BP the obese rats demonstrated slightly greater pressor responses to phenylephrine than their lean littermates. The differences between the vascular responses were statistically significant at the 10, 30 and 100 $\mu\text{g kg}^{-1}$ phenylephrine doses ($P<0.05$, $P<0.05$ and $P<0.005$ respectively by unpaired t-test), despite the small number of animals tested. The vascular responses of the two phenotypes to i.v. phenylephrine are represented graphically in **Figure 3.1.1**.

Conclusions

Earlier BP measurements using radio-telemetry techniques carried out, in our work group, on lean and obese AP Zucker rats demonstrated that the obese rats were hypertensive relative to their lean counterparts (these results are summarised in **Appendix 1**).

Figure 3.1.1. The Blood Pressure Responses of Conscious Lean and Obese AP Zucker Rats to i.v. Phenylephrine

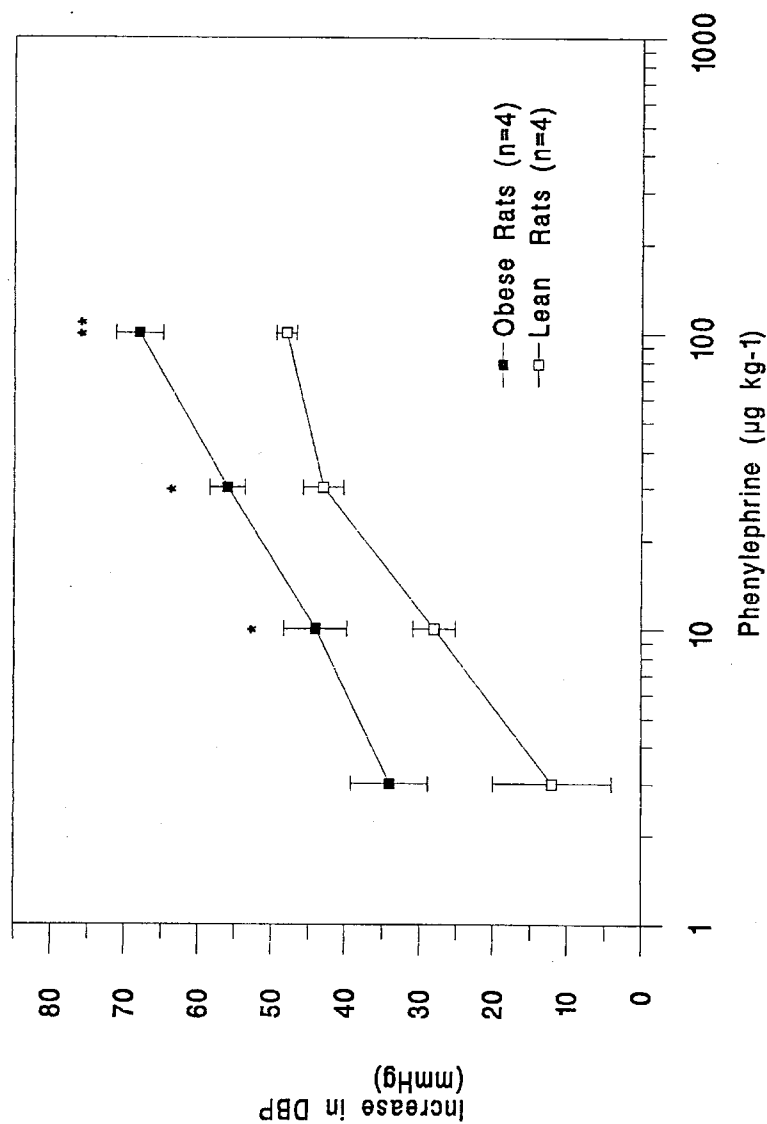


Figure 3.1.1 Groups of AP Zucker rats were cannulated under anaesthesia and allowed to recover. The increases in diastolic blood pressure (mmHg) due to i.v. phenylephrine in small groups of conscious obese (n=4) and lean (n=3) AP Zucker rats on the day following surgery are illustrated. Significant differences were found at the 10, 30 and 100 µg kg⁻¹ doses ($P < 0.05$, $P < 0.05$ and $P < 0.01$ respectively by unpaired t-test).

These data support the literature data which indicate that the obese Zucker is hypertensive compared to its lean counterpart. Due to the fact that the data was obtained from less stressed animals, the radio-telemetry data (considered along with the literature precedent) indicated that the conscious BP measurement obtained from the experiment described above may have given a false impression of the BP levels of the lean and obese animals (especially as it was found that the obese rat had a greater vascular reactivity than its lean counterpart).

3.1.2 Experiment in Anaesthetised Rats

Introduction

The experiment described in section 3.1.1 demonstrated that factors such as surgical stress may have important effects on the haemodynamic profile of the Zucker rats tested. In order to examine the vascular responses of the two Zucker rat phenotypes without the stress caused by recovery surgery, animals were cannulated and their vascular responses assessed under anaesthesia without recovery.

Rats were anaesthetised with Intraval at 30 mg kg⁻¹ via the i.p. route. The carotid artery and jugular vein were cannulated (as described in the methodology for the conscious experiment) but there was no need for the cannulae to be exteriorised as no recovery was to be allowed. The BP and heart rate of the animals were monitored to assess the depth of anaesthesia. The assessment of the vascular responses of the animals to pressor agents was carried out in the same manner as described in the pithed animal methodology (section 2.3.2).

This study used lean and obese AP Zucker male rats of the same age as those used in the conscious experiment described above (section 3.1.1). The pressor agents used in this experiment were phenylephrine at 3, 10, 30 and 100 µg kg⁻¹ and angiotensin II at 10, 30, 100, 300 and 1000 ng kg⁻¹.

Results

During the course of the experiment it was found necessary to continually provide more and more anaesthetic to the obese rats to maintain an adequate depth of anaesthesia. The lean rats only required the initial anaesthetic dose to maintain a good depth of anaesthesia throughout the entire experiment. This difference between the two phenotypes was probably due to the large adipose content in the obese animals and the lipophilicity of the Intraval anaesthetic used. Due to this problem it may have been the case that the two phenotypes were at slightly different depths of anaesthesia during the experiment and would probably have had different concentrations of anaesthetic in the circulation which may have influenced the results obtained from the dosing of the two pressor agents.

The difference between the obese rat response and the lean rat response to phenylephrine were not statistically significant due to the large variation in responses of the obese animals. The vascular responses of the lean and obese rats to phenylephrine dosing are represented graphically in **Figure 3.1.2**. The vascular responses of the lean and obese rats to angiotensin II dosing were significantly different, especially at the lower concentrations of the pressor agent, and are represented graphically in **Figure 3.1.3**.

Conclusions

Despite the problems found in maintaining a stable depth of anaesthesia, the obese animals had a consistently greater vascular response to both phenylephrine and angiotensin II dosing than was found in the lean animals (although this difference was not statistically significant when phenylephrine was used). This difference in vascular reactivity was similar to that found in the experiment on conscious animals described previously.

Figure 3.1.2 The Blood Pressure Responses of Anaesthetised Lean and Obese AP Zucker Rats to i.v. Phenylephrine

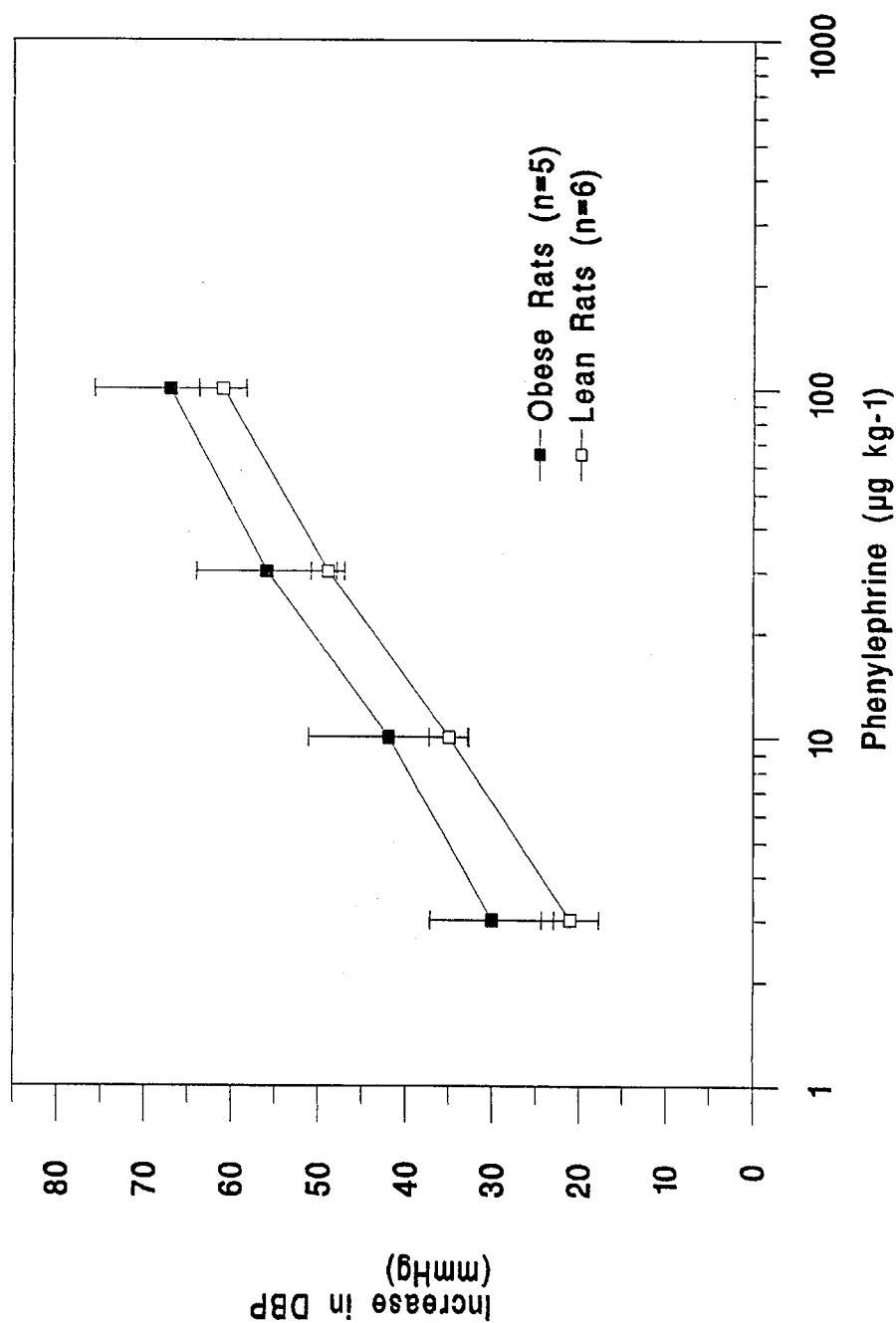


Figure 3.1.2 Groups of AP Zucker rats were cannulated under anaesthesia. The increases in diastolic blood pressure (mmHg) due to i.v. phenylephrine in groups of anaesthetised obese (n=5) and lean (n=6) AP Zucker rats are illustrated. No significant differences were found between the two groups.

Figure 3.1.3 The Blood Pressure Responses of Anaesthetised Lean and Obese AP Zucker Rats to i.v. Angiotensin II

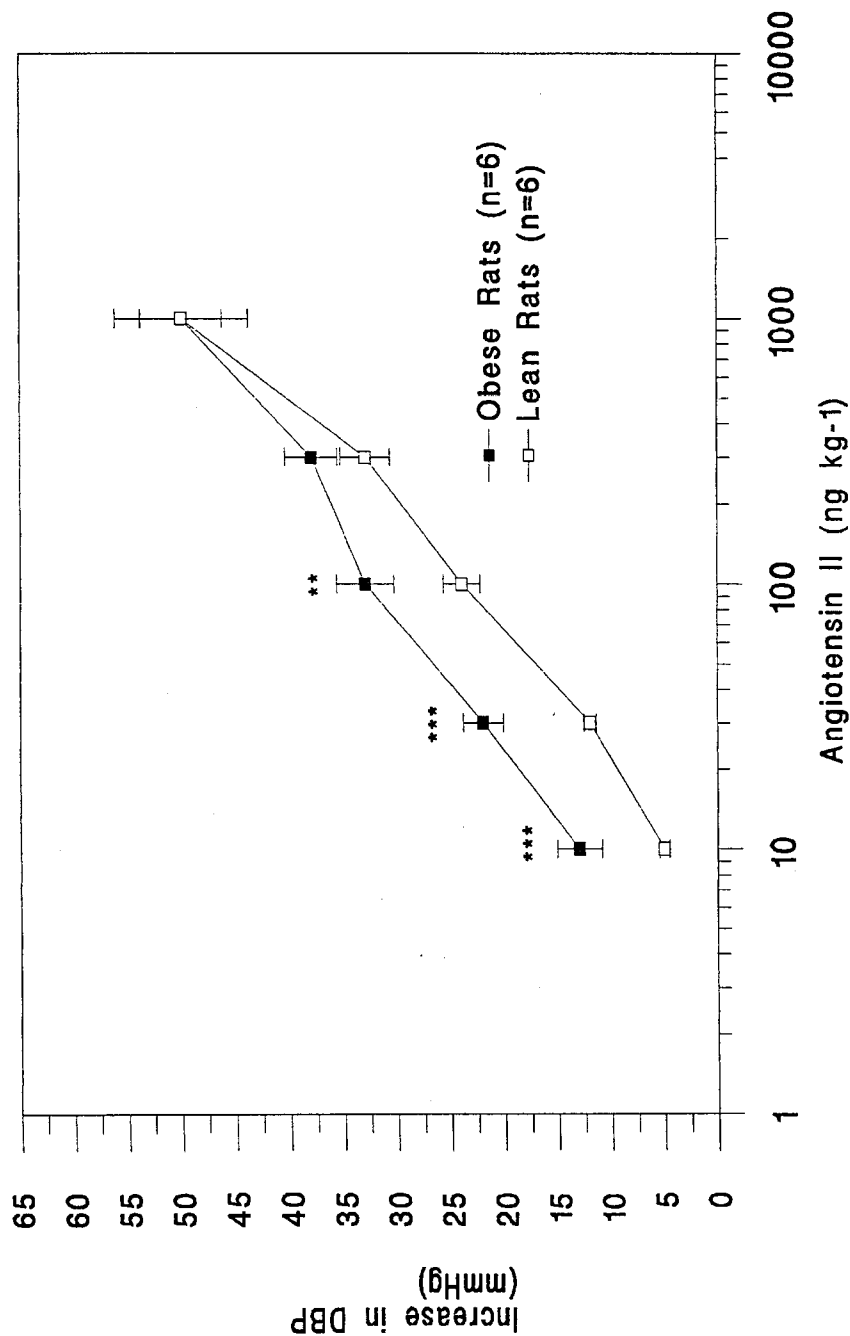


Figure 3.1.3 Groups of AP Zucker rats were cannulated under anaesthesia. The increases in diastolic blood pressure (mmHg) due to i.v. angiotensin II in groups of anaesthetised obese (n=6) and lean (n=6) AP Zucker rats are illustrated. Significant differences were found between the two groups at the 10, 30 and 100 ng kg⁻¹ doses ($P < 0.001$, $P < 0.001$ and $P < 0.01$ respectively by unpaired t-test).

3.1.3 Experiment in Pithed Rats

Introduction

Due to the different anaesthetic handling properties of the two AP Zucker rat phenotypes outlined above, it was decided that it was necessary to use a preparation in which the influence of anaesthesia was reduced. The experiment in conscious rats, as described in section 3.1.1 above, demonstrated that animals used in a recovery experiment may be affected by surgical stress. The only other type of preparation in which the animals are relatively free of anaesthesia is a pithed preparation. The full methodology for the pithed rat experiment is described in the materials and methods chapter (section 2.1).

In this initial experiment groups of four male lean and four obese AP Zucker rats aged three and a half months were used. The pressor agents used in this experiment were phenylephrine at doses of 3, 10, 30 and 100 $\mu\text{g kg}^{-1}$ and angiotensin II at 10, 30, 100, 300 and 1000 ng kg^{-1} .

Results

It was found that the obese rats demonstrated greater vascular responses to both phenylephrine and angiotensin II than the lean rats. The vascular responses of the lean and obese rats to phenylephrine are represented graphically in **Figure 3.1.4**. The vascular responses of the lean and obese rats to angiotensin II dosing are represented graphically in **Figure 3.1.5**.

Conclusions

The pithed experiment provided similar data to that from the conscious and anaesthetised experiments described above in that a greater vascular responsiveness was found in the obese Zucker rat compared to its lean counterpart. It was assumed that the results obtained

Figure 3.1.4. The Blood Pressure Responses of Pithed Lean and Obese AP Zucker Rats to i.v. Phenylephrine

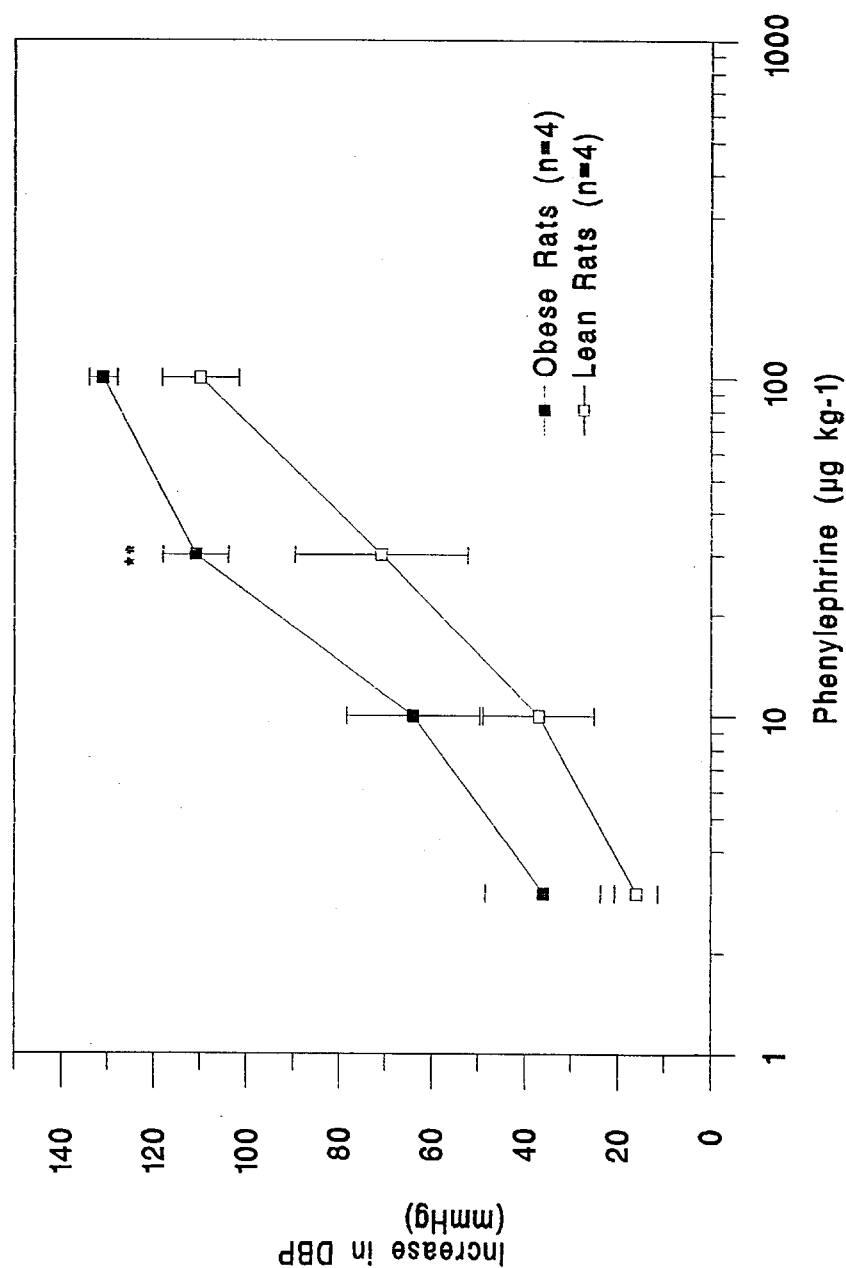


Figure 3.1.4 Groups of AP Zucker rats were pithed under anaesthesia. The increases in diastolic blood pressure (mmHg) due to i.v. phenylephrine in groups of obese (n=4) and lean (n=4) AP Zucker rats are illustrated. A significant difference was found between the two groups at the 30 $\mu\text{g kg}^{-1}$ dose ($P < 0.01$ by unpaired t-test).

Figure 3.1.5 The Blood Pressure Responses of Pithed Lean and Obese AP Zucker Rats to i.v. Angiotensin II

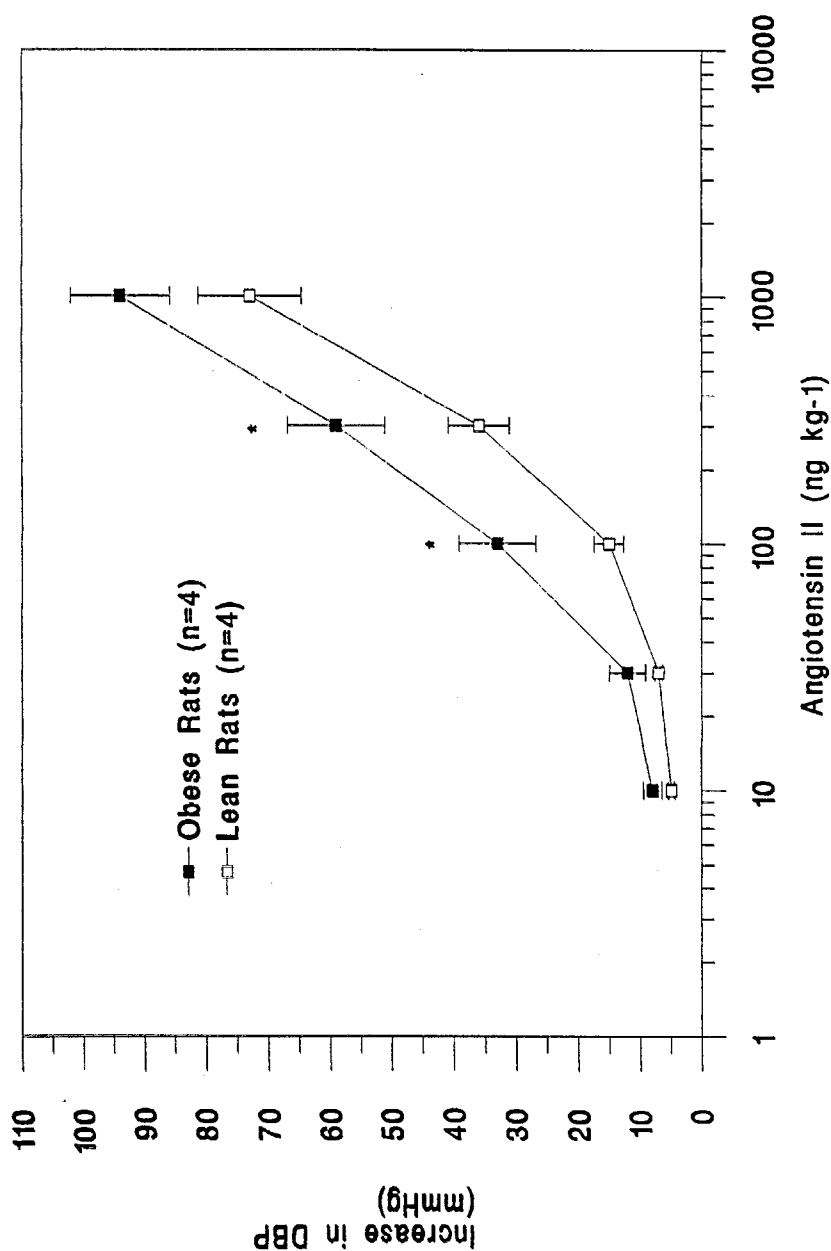


Figure 3.1.5 Groups of AP Zucker rats were pithed under anaesthesia. The increases in diastolic blood pressure (mmHg) due to i.v. angiotensin II in groups of obese (n=4) and lean (n=4) AP Zucker rats are illustrated. Significant differences were found between the two groups at the 100 and 300 ng kg⁻¹ dose (both $P < 0.05$ by unpaired t-test).

from the pithed preparation were relatively free of the influence of surgical stress and different depths of anaesthesia in both of the Zucker rat phenotypes.

The experiments described in the previous sections demonstrated that two main factors must be taken into account when investigating the haemodynamic responses of the lean and obese AP Zucker rat, these being surgical stress and anaesthesia. It was concluded that the pithed methodology was the best to use when comparing the vascular reactivities of the two AP Zucker phenotypes.

(3.2) The Effect of a High Fat Diet on the Vascular Reactivity of the AP Wistar Rat.

Introduction

Literature reports have indicated that a high fat diet can induce an insulin resistant state in experimental animals (see introduction). The high fat diet used (composed of 60 % ground normal diet with 30 % lard and 10 % caesin) has been shown to increase both plasma insulin and glucose in AP Wistar rats. Plasma insulin and glucose measurements from Wistar rats fed this diet for a period of four weeks indicated that a degree of IR was induced by this diet (typical results are summarised below). These results were obtained by other workers at Zeneca using AP strain Wistar rats.

Table 3.2.1 The Effects of High Fat Feeding on Plasma Insulin and Plasma Glucose Levels in AP Wistar Rats

Plasma insulin Levels (ng ml⁻¹)

	Sample (n=)	mean	s.e.
Control	4	0.61	0.15
Week 1	10	1.92	0.28
Week 2	6	1.86	0.52
Week 3	5	3.02	0.79
Week 4	6	2.64	0.69

Plasma Glucose Levels (mM)

	Sample (n=)	mean	s.e.
Control	4	7.05	0.45
Week 1	10	8.17	0.41
Week 2	6	8.32	0.36
Week 3	5	9.22	0.17
Week 4	6	12.45	1.71

Since the high fat diet has been shown to increase plasma insulin and glucose levels it was decided to test the ability of the diet to alter the vascular reactivity of the AP wistar rat. Male AP Wistar rats of three months of age were used. One group of rats was placed on the high fat diet for a period of six weeks while a second group was placed on normal

ground diet prior to the assessment of vascular reactivity to phenylephrine. The assessment of vascular reactivity was carried out in an identical manner to that in the pithed Zucker rat experiments described previously.

Results

It was found that only very small differences existed between the vascular responses of the AP Wistar rats fed a control diet and those fed the high fat diet. A statistically significant difference was found between the control fed and high fat fed groups ($P < 0.05$ by unpaired t-test) only at the $100 \mu\text{g kg}^{-1}$ phenylephrine dose. These results are expressed graphically as **Figure 3.2.1**.

Conversely it was found that the group of Wistar rats fed the high fat diet demonstrated slightly smaller angiotensin II induced BP increases when compared to the control group fed a normal diet. This small difference was only statistically significant at the 100 ng kg^{-1} dose ($P < 0.05$ by unpaired t-test). These results are presented graphically as **Figure 3.2.2**.

Conclusions

It was found that the imposition of a high fat diet did not increase the vascular responsiveness of the AP wistar rat. Other experiments using the same high fat diet regime on AP Wistar rats implanted with radio-telemetry equipment was carried out by other workers at Alderley Park. These experiments demonstrated that the diet did induced greater weight gain in the fat fed rats (compared to a control group fed a normal diet) but that no blood pressure increase was induced by the diet.

Figure 3.2.1 The Effect of a High Fat Diet on the Blood Pressure Responses of AP Wistar Rats to i.v. Phenylephrine

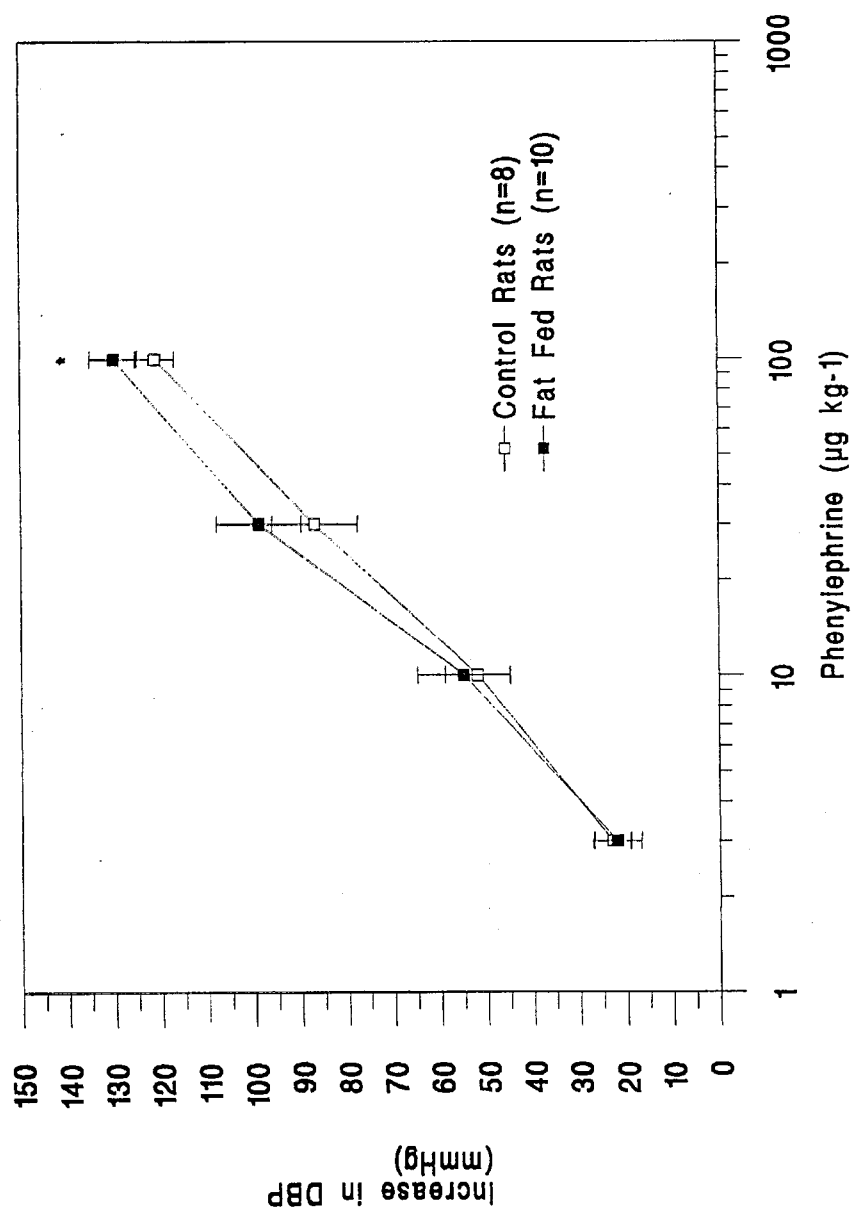


Figure 3.2.1 A group of AP Wistar rats was fed a high fat diet for a period of six weeks and then pithed under anaesthesia. The increases in diastolic blood pressure (mmHg) due to i.v. phenylephrine in the fat fed group (n=10) and a second group fed a normal diet (n=8) is presented above. A significant difference was found between the two groups at the 100 $\mu\text{g kg}^{-1}$ dose ($P < 0.05$ by unpaired t-test).

Figure 3.2.2 The Effect of a High Fat Diet on the Blood Pressure Responses of AP Wistar Rats to i.v. Angiotensin II

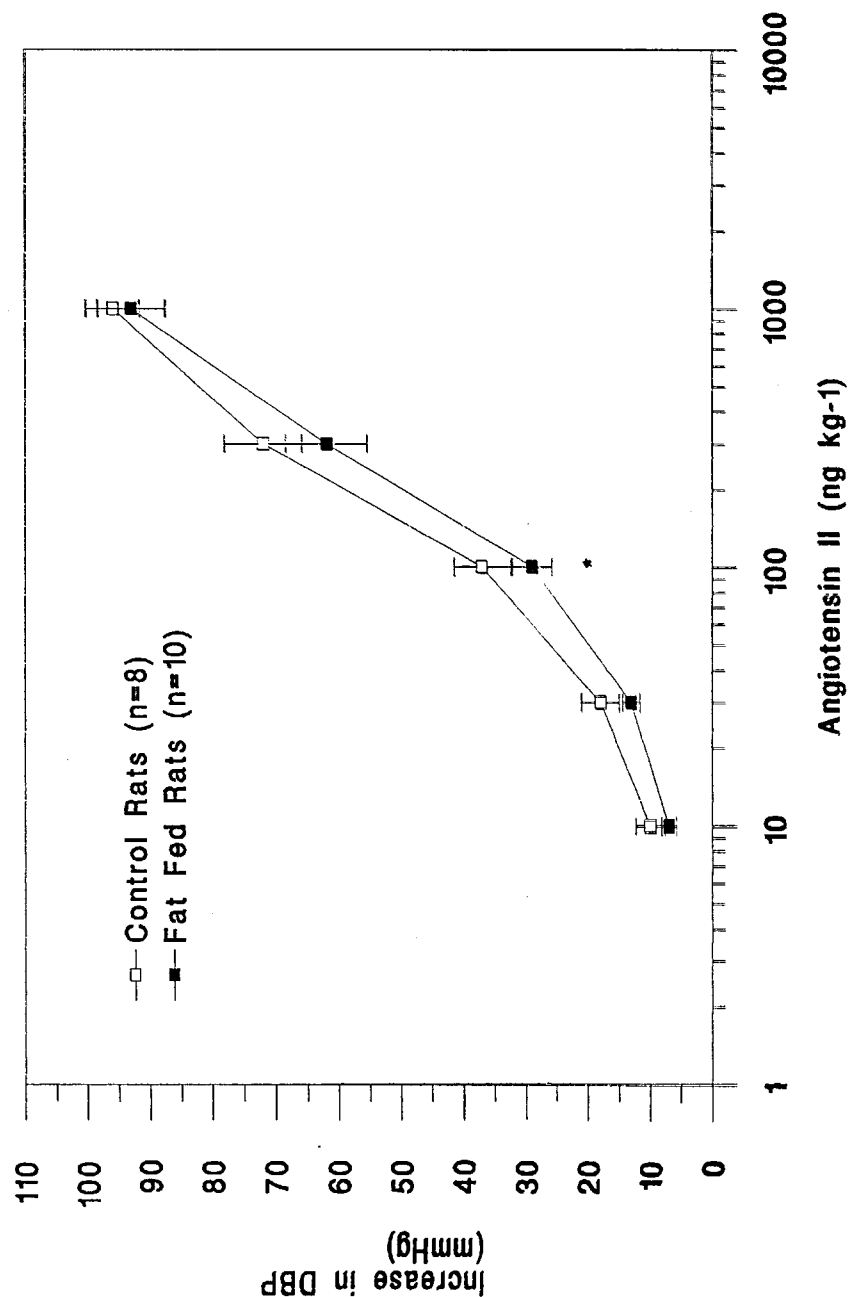


Figure 3.2.2 A group of AP Wistar rats was fed a high fat diet for a period of six weeks and then pithed under anaesthesia. The increases in diastolic blood pressure (mmHg) due to i.v. angiotensin II in the fat fed group (n=10) and a second group fed a normal diet (n=8) is presented above. A significant difference was found between the two groups at the 100 ng kg⁻¹ dose ($P < 0.05$ by unpaired t-test).

(3.3) The Effects of Pioglitazone on The Vascular Reactivity of the AP Zucker Rat.

Introduction

In the initial experiments carried out on the AP Zucker rat (outlined above in section 3.1), the obese AP strain Zucker rat consistently demonstrated a greater vascular reactivity than its lean littermate. Literature reports (as described in the introduction) indicate that IR may be linked to haemodynamic changes. In order to further investigate the link between IR and vascular reactivity in the obese Zucker rat, it was decided to investigate whether an insulin sensitising agent could affect the haemodynamic profile of the animal.

The insulin sensitising agent chosen was pioglitazone, an orally active thiazolidenedione compound. The dosing regime chosen for the following experiments was based on results from the dosing of the compound to telemetered AP obese Zucker rats (see **Appendix 1** for a full review of this experiment).

Experiment One

Introduction

A sighting experiment was carried out in small groups of lean and obese male AP Zucker rats of approximately six months of age. Five obese and five lean rats were treated with 30 mg kg⁻¹ pioglitazone once daily p.o. for seven days. In addition, five lean and five obese animals were dosed in an identical manner except polysorbate (vehicle) alone was used.

Results

As found in previous experiments, the obese Zucker rats treated with vehicle demonstrated greater vascular responses to phenylephrine than the lean rats treated with vehicle. A significant difference was found between these groups at the 100 µg kg⁻¹ phenylephrine dose ($P < 0.05$ by unpaired t-test). The obese rats treated with pioglitazone

demonstrated significantly smaller responses to phenylephrine than obese rats treated with vehicle alone ($P < 0.05$ at both the 3 and 10 $\mu\text{g kg}^{-1}$ doses and $P < 0.01$ at the 30 and 100 $\mu\text{g kg}^{-1}$ doses). The vehicle treated and pioglitazone treated lean rats demonstrated no significant differences in their responses to phenylephrine. These results are presented graphically as **Figure 3.3.1**.

Conclusions

Despite the small group sizes used in this sighting study two observations were made. Firstly, the greater reactivity of the obese Zucker compared to its lean littermate was again present. Secondly, statistically significant differences were found between the obese rats treated with pioglitazone and those treated with vehicle alone.

Figure 3.3.1 The Effect of Pioglitazone on the Blood Pressure Responses of Male Lean and Obese AP Zucker Rats to Phenylephrine

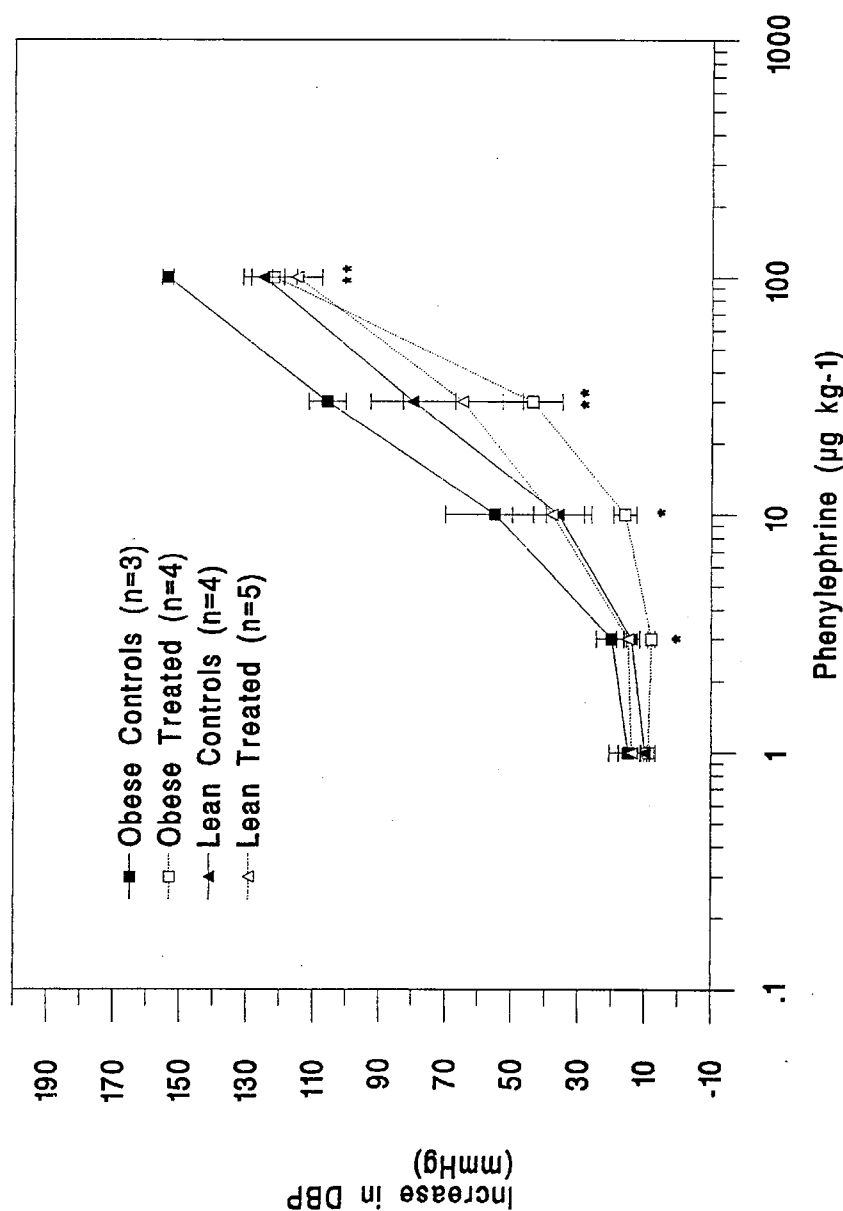


Figure 3.3.1 Groups of lean and obese male AP Zucker rats were treated with 30 mg kg^{-1} pioglitazone p.o. or vehicle once daily for a period of seven days. The rats were then pithed. The increases in diastolic blood pressure (mmHg) due to i.v. phenylephrine in obese drug treated ($n=4$), obese vehicle treated ($n=3$), lean drug treated ($n=4$) and lean vehicle treated ($n=5$) are illustrated. A significant difference was found between the two vehicle treated groups at the $100 \text{ } \mu\text{g kg}^{-1}$ dose ($P<0.05$ by unpaired t-test). Significant differences were found between drug and vehicle treated obese rats at the 3, 10, 30, and $100 \text{ } \mu\text{g kg}^{-1}$ doses ($P<0.05$, $P<0.01$ and $P<0.01$ respectively).

Experiment Two

Introduction

In the second experiment, due to a brief unavailability of male rats, a larger pioglitazone dosing study was conducted in groups of female lean and obese AP Zucker rats. The female rats were slightly younger than the male rats used in the sighting experiment described above (being aged approximately four months). Ten obese rats were dosed with pioglitazone (using the same protocol as described above) as were ten lean rats. Ten obese and ten lean rats were dosed with polysorbate vehicle alone.

Results

As found in the previous experiment with male rats, the female obese Zucker rats treated with vehicle demonstrated greater vascular responses to phenylephrine doses than the lean rats treated with vehicle. These differences were statistically significant at the 3, 10 and 100 $\mu\text{g kg}^{-1}$ phenylephrine doses ($P < 0.05$, $P < 0.01$ and $P < 0.05$ respectively by unpaired t-test). The female obese rats treated with pioglitazone demonstrated significantly smaller responses to phenylephrine than obese rats treated with vehicle alone ($P < 0.05$ at the 10 and 100 $\mu\text{g kg}^{-1}$ phenylephrine doses and $P < 0.01$ at the 30 $\mu\text{g kg}^{-1}$ dose by unpaired t-test). The vehicle treated and pioglitazone treated lean rats demonstrated no significant differences in their responses to phenylephrine. These results are presented graphically as **Figure 3.3.2**.

Conclusions

The results obtained from this larger group of female rats agreed with the results from the smaller study using male rats in that a reactivity differential between the obese and lean animals was again present and pioglitazone significantly reduced the enhanced reactivity of the obese animal.

Figure 3.3.2 The Effect of Pioglitazone on the Vascular Reactivity of Female Lean and Obese Zucker Rats

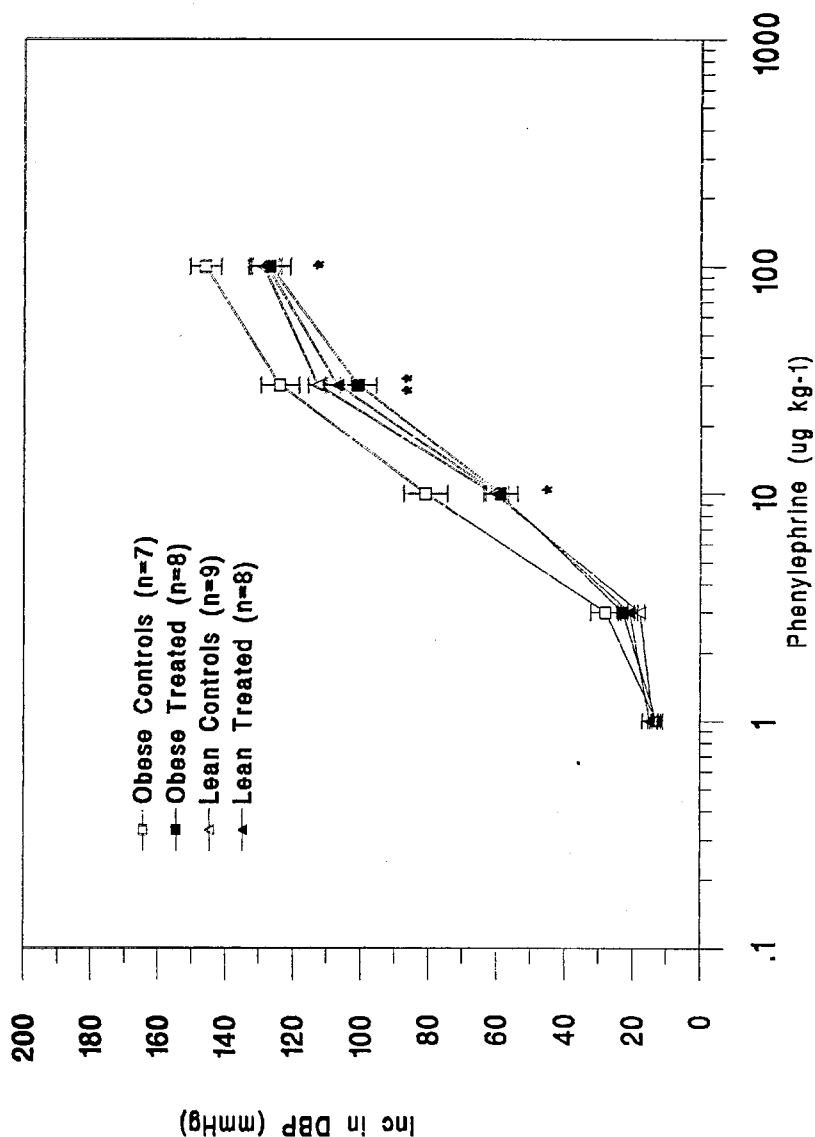


Figure 3.3.2 Groups of lean and obese female AP Zucker rats were treated with 30 mg kg⁻¹ pioglitazone or vehicle p.o. once daily for a period of seven days. The rats were then pithed. The increases in diastolic blood pressure (mmHg) due to i.v. phenylephrine in obese drug treated (n=8), obese vehicle treated (n=7), lean drug treated (n=8) and lean vehicle treated (n=9) are illustrated. Significant differences were found between the two vehicle treated groups at the 3, 10 and 100 µg kg⁻¹ doses ($P<0.05$, $P<0.01$ and $P<0.05$ respectively by unpaired t-test). Significant differences were found between drug and vehicle treated obese rats at the 10, 30, and 100 µg kg⁻¹ doses ($P<0.05$, $P<0.01$ and $P<0.05$ respectively by unpaired t-test).

Experiment Three

Introduction

When a larger group of male rats became available a larger pioglitazone dosing study was carried out to confirm the observations made from the sighting study and the female rat study. In this study ten obese animals were dosed with pioglitazone and a further ten with polysorbate vehicle alone.

Results

The male obese rats treated with pioglitazone demonstrated significantly smaller responses to phenylephrine than obese rats treated with vehicle alone ($P < 0.01$ at the 3 and 10 $\mu\text{g kg}^{-1}$ doses and $P < 0.05$ at the 100 $\mu\text{g kg}^{-1}$ phenylephrine dose by unpaired t-test). These results are presented graphically as **Figure 3.3.3**.

Conclusions

The results from this larger study using male rats agreed with both the smaller male study (described in section 3.3.1.) and the study using female rats (described in section 3.3.2.) in that pioglitazone again produced a significant reduction in the vascular reactivity of the obese AP Zucker rat. In the first two experiments the pioglitazone treated obese rats demonstrated a very similar level of reactivity to the vehicle and pioglitazone treated lean Zucker rats. One implication that may be drawn from these experiments is that pioglitazone is able to reduce hyper-reactivity in the insulin resistant obese Zucker rat by means of the insulin sensitising properties of the agent.

Figure 3.3.3 The Effect of Pioglitazone on the Blood Pressure Responses of Obese Male Zucker Rats to Phenylephrine

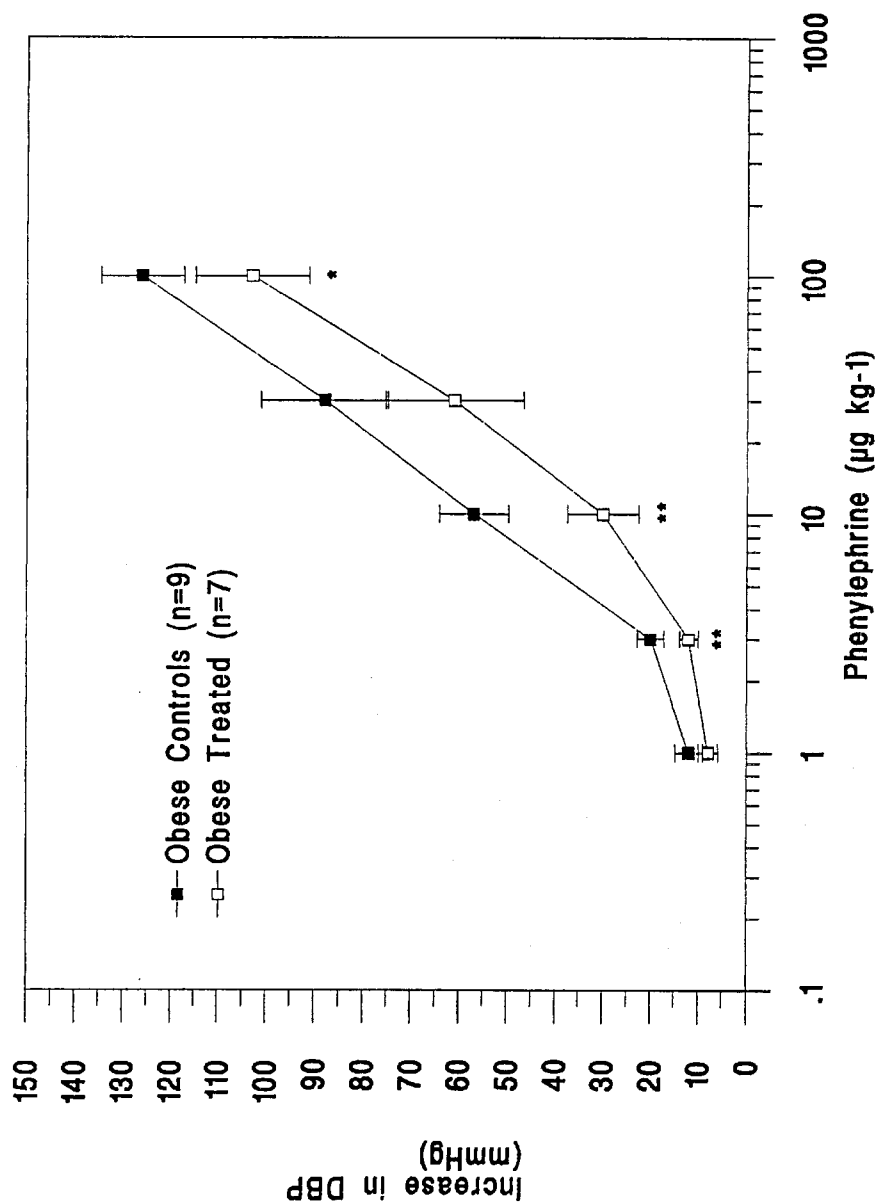


Figure 3.3.3 Groups of obese male AP Zucker rats were treated with 30 mg kg⁻¹ pioglitazone or vehicle p.o. once daily for a period of seven days. The rats were then pithed. The increases in diastolic blood pressure (mmHg) due to i.v. phenylephrine in drug treated (n=7) and vehicle treated (n=9) rats are illustrated. Significant differences were found between the two groups at the 3, 10 and 100 µg kg⁻¹ doses ($P<0.01$, $P<0.01$ and $P<0.05$ respectively by unpaired t-test).

(3.4) The Effects of Pioglitazone Treatment on the Vascular Reactivity of AP Wistar and AP SH Strain Rats.

Introduction

The results obtained from the pioglitazone treatment of the obese and lean AP Zucker rat suggested that pioglitazone had selective effects in the obese rat. The main implication that may be drawn from this observation, given that pioglitazone is a known insulin sensitising agent, is that the fall in vascular reactivity seen in the obese AP Zucker rat is as a result of an increase in insulin sensitivity (i.e., due to a decrease in IR). The inference from this argument would be that the vascular reactivity of the lean rats was unaffected due to the normal insulin sensitivity of the lean phenotype.

In order to assess the selectivity of action of the pioglitazone treatment it was decided to test the ability of the agent to alter the vascular responses of two alternative rat strains, the normotensive AP Wistar rat and the hypertensive (and hyper-reactive) AP SH rat.

Both the AP Wistar and AP SH strain rats used in this study were male and aged approximately four months. As in previous experiments, one group of the animals was dosed with pioglitazone once daily for seven days and another group was dosed in the same manner with polysorbate vehicle alone. At the end of the dosing period the vascular reactivity of the two groups was assessed in the same manner as described previously.

Results

The vascular reactivity of the AP Wistar rat was found to be very similar to that of the obese AP Zucker rat. The pioglitazone dosing produced no significant decrease in the vascular reactivity of the AP Wistar rat. The vascular reactivities of the control and pioglitazone treated Wistar rats are presented as **Figure 3.4.1**.

As expected, the vascular reactivity of the control treated AP SH rats was greater than that of the AP Wistar rats. Despite this greater reactivity, however, no difference could be

Figure 3.4.1 The Effect of Pioglitazone on the Blood Pressure Response of Male AP Wistar Rats to Phenylephrine

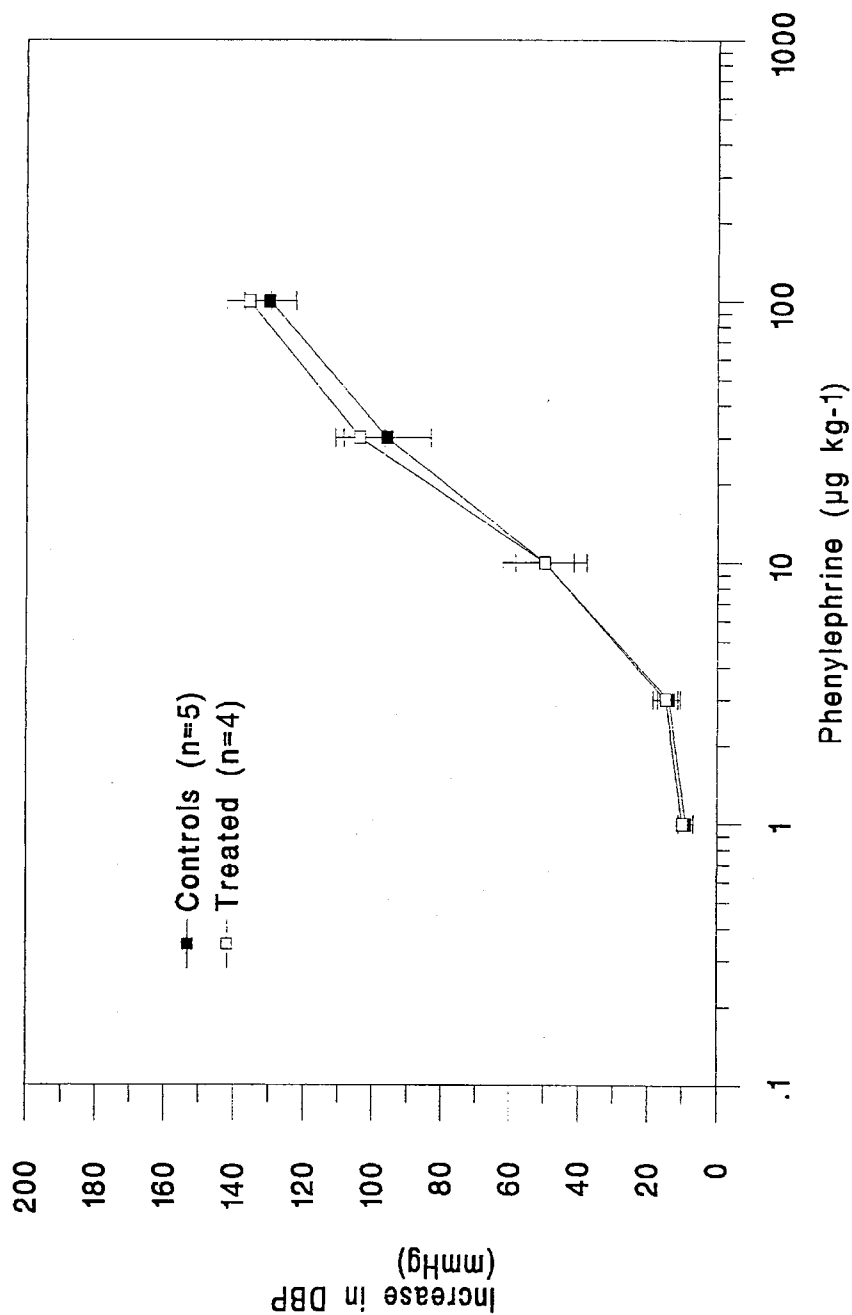


Figure 3.4.1 Groups of male AP Wistar rats were treated with 30 mg kg^{-1} pioglitazone or vehicle p.o. once daily for a period of seven days. The rats were then pithed. The increases in diastolic blood pressure (mmHg) due to i.v. phenylephrine in drug treated ($n=4$) and vehicle treated ($n=5$) rats are illustrated. No significant differences were found between the two groups.

seen between the control and pioglitazone treated SH rats. These results are presented graphically as **Figure 3.4.2**.

Conclusions

The experiments described in this section demonstrated that the same pioglitazone treatment regime that was effective in reducing the vascular reactivity of the obese AP Zucker rat in previous experiments was ineffective in altering the vascular responses of the two rat strains tested.

Figure 3.4.2 The Effect of Pioglitazone on the Blood Pressure Responses of AP SH Rats to Phenylephrine

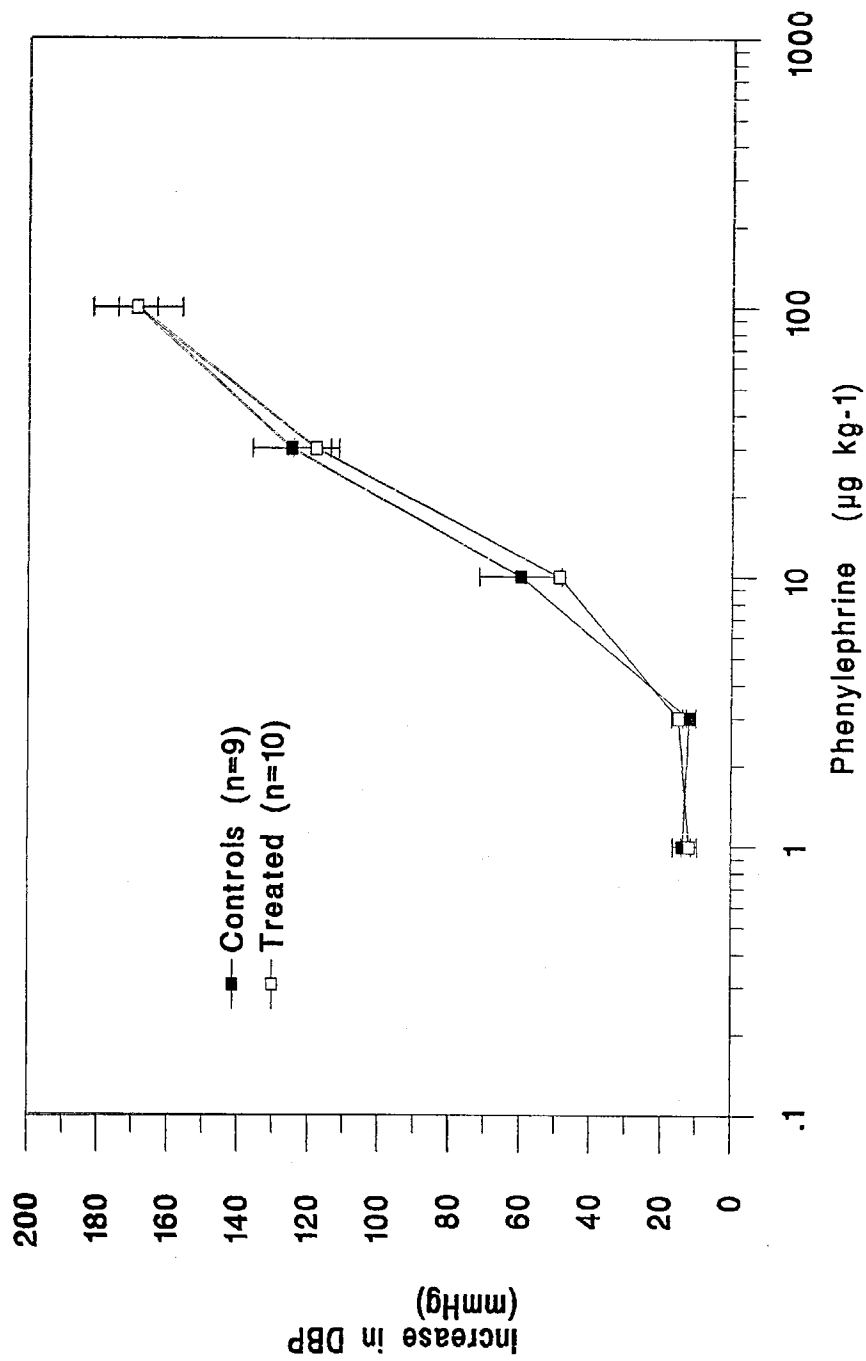


Figure 3.4.2 Groups of male AP SH rats were treated with 30 mg kg⁻¹ pioglitazone or vehicle p.o. once daily for a period of seven days. The rats were then pithed. The increases in diastolic blood pressure (mmHg) due to i.v. phenylephrine in drug treated (n=10) and vehicle treated (n=9) rats are illustrated. No significant differences were found between the two groups.

(3.5) The Effects of Pioglitazone on the Blood Pressure of the AP SH Rat as Measured by a Radio-telemetry System

Introduction

In the experiments described above and in other experiments carried out within our work group, pioglitazone has been shown to decrease both the vascular reactivity and the BP of the obese AP Zucker rat (see **Appendix 1**). In contrast, the agent has been shown to be ineffective in altering the vascular reactivity of the lean AP Zucker rat, the AP Wistar rat and the AP SH rat.

In an experiment carried out within our work group using a radio-telemetry system, as described in **Appendix 1**, pioglitazone was shown to decrease the BP of the obese rat and, to a much lesser effect, the lean AP Zucker rat. It was not clear from this experiment whether pioglitazone produced the greater effect in the obese animal due to a selective mechanism (i.e., in altering the insulin resistant state of the animal) or merely due to the fact that the obese animal had a higher starting BP. It was decided to test the ability of pioglitazone to affect the BP of the AP SH rat (which consistently demonstrates a much higher basal BP than the obese AP Zucker rat).

Results

Due to the normal daily activity pattern of the rats tested, the results obtained from this experiment have been divided into day time and night time means. Significant differences were found between the systolic, mean and diastolic BP values (both during the day and night time halves of the daily light cycle) observed after four days of polysorbate (vehicle) dosing and those observed after four days of pioglitazone dosing (by paired t-test). Data from the four day time-point for both the vehicle and drug treatment periods were studied as they were comparable to the time-points measured in the study of pioglitazone treatment in obese and lean AP Zucker rats (see **Appendix 1**).

Table 3.5.1 The Effect of Oral Pioglitazone at 30 mg kg⁻¹ day⁻¹ on the Blood Pressure, Heart Rate and Activity Level of AP SH rats (n=4)

Day Time Means

	Systolic BP (mmHg)	Diastolic BP (mmHg)	Heart Rate (bpm)	Activity Level (units)
Control Period	208	145	310	15
Treated Period	201	138	306	14

Night Time Means

	Systolic BP (mmHg)	Diastolic BP (mmHg)	Heart Rate (bpm)	Activity Level (units)
Control Period	216	151	351	36
Treated Period	204	139	351	31

Please refer to **Figure 3.5.1** and **Figure 3.5.2** to view the daily mean results from this experiment presented graphically.

Conclusions

In this experiment pioglitazone dosing produced small but significant decreases in both day time and night time blood pressure levels in the AP SH rats tested. No significant effects were seen on the heart rate of the animals tested.

Figure 3.5.1 The Effect of Pioglitazone at 30 mg kg⁻¹ Daily (p.o.) on Day Time Blood Pressure and Heart Rate in the AP SH Rat (n=4)

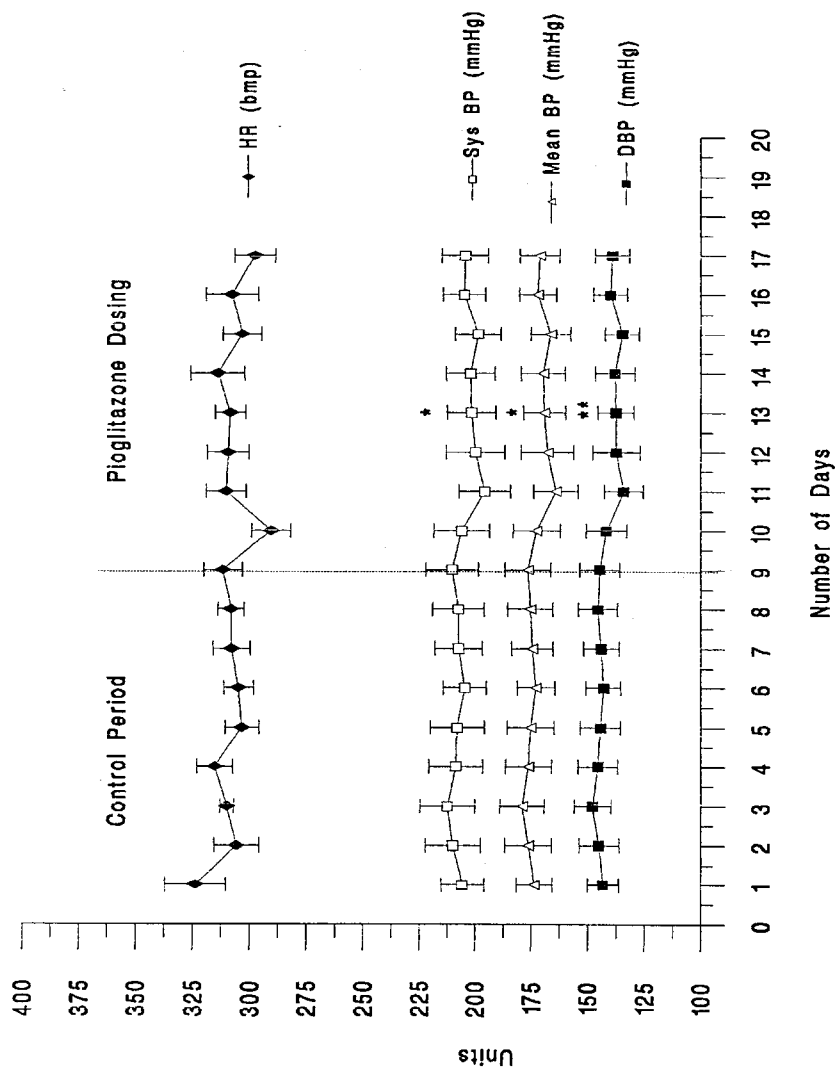


Figure 3.5.1 A group of male AP SH rats, implanted with a radio-telemetry blood pressure measuring unit, were dosed with polysorbate for seven days. Following this control period the rats were treated with 30 mg kg⁻¹ pioglitazone p.o. once daily for a period of seven days. The day time blood pressure and heart rate values are illustrated. On the fourth day of pioglitazone treatment significant falls in systolic, mean and diastolic blood pressures were observed ($P < 0.05$, $P < 0.05$ and $P < 0.01$ respectively by paired t-test) when compared to the values after four days of vehicle treatment.

Figure 3.5.2 The Effect of Pioglitazone at 30 mg kg⁻¹ Daily (p.o.) on Night Time Blood Pressure and Heart Rate in the AP SH Rat (n=4)

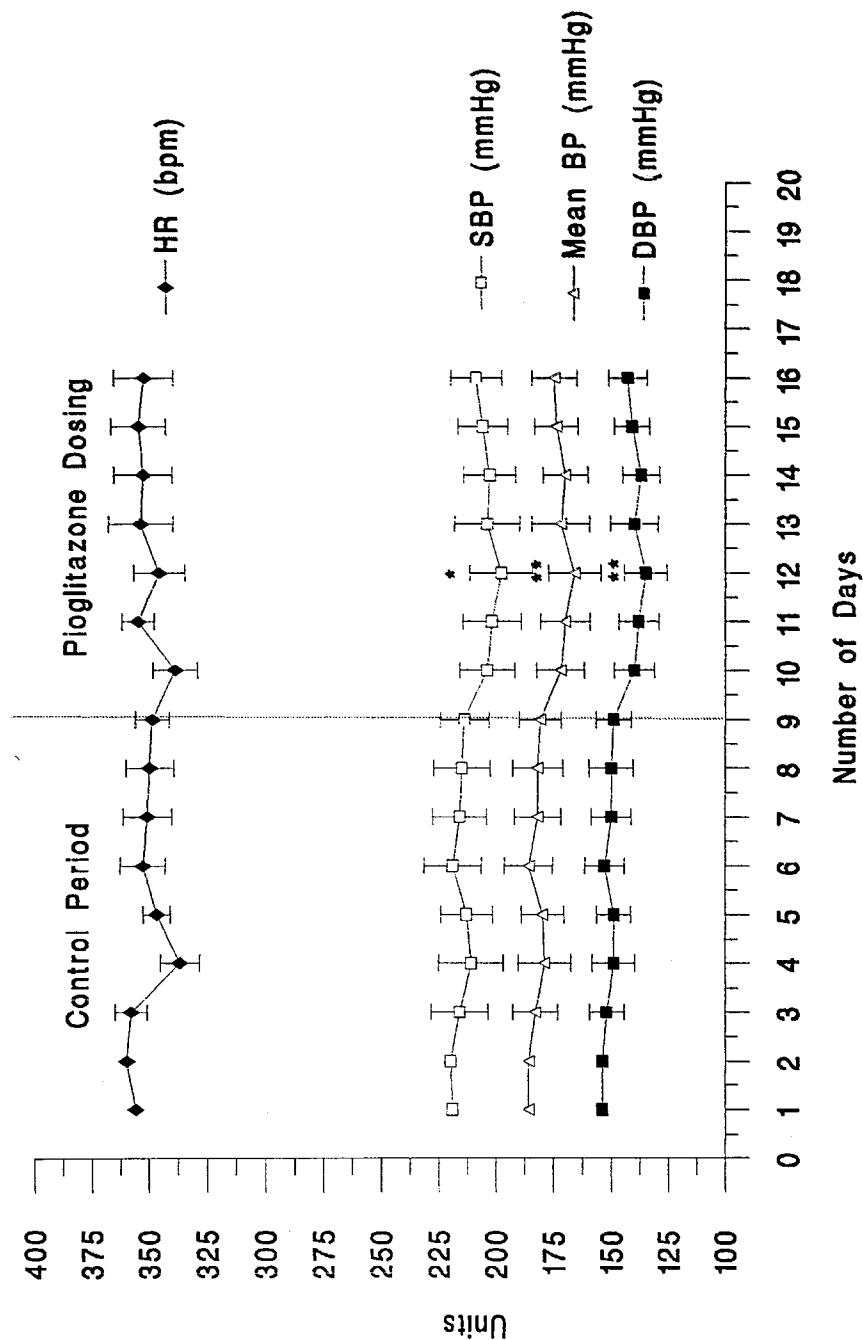


Figure 3.5.2 A group of male AP SH rats, implanted with a radio-telemetry blood pressure measuring unit, were dosed with polysorbate for seven days. Following this control period the rats were treated with 30 mg kg⁻¹ pioglitazone p.o. once daily for a period of seven days. The night time blood pressure and heart rate values are illustrated. On the fourth day of pioglitazone treatment significant falls in systolic, mean and diastolic blood pressures were observed ($P<0.05$, $P<0.01$ and $P<0.01$ respectively by paired t-test) when compared to the values after four days of vehicle treatment.

(3.6) The Effects of Two Other Insulin Sensitising Agents on the Vascular Reactivity of the AP Zucker Rat.

Introduction

Previous experiments, described above, have demonstrated that the insulin resistant obese AP Zucker rat is hypertensive and has an enhanced vascular reactivity when compared to its lean littermate. Furthermore, it has been demonstrated that pioglitazone, an orally active insulin sensitising agent, reduced both the BP and vascular reactivity of the obese rat. One implication that can be drawn from this observation is that pioglitazone was effective in reducing vascular reactivity and BP as a result of its insulin sensitising properties (i.e., that the haemodynamic changes seen in the obese rat were the result of an improvement in insulin sensitivity).

In order to further investigate the link between the insulin resistant state and vascular reactivity it was decided to dose two other insulin sensitising compounds (unrelated to pioglitazone) to groups of obese and lean AP Zucker rats and assess the effect of the treatment on the vascular reactivity of the animals.

With the literature reports in mind (as outlined in the introduction section), it was decided to use ZD2079 (a β -3 adrenoceptor agonist) and vandyl sulphate (a vanadium containing compound) as alternative insulin sensitising treatments in obese AP Zucker rats and to assess the effects of the two treatments on the vascular reactivity of the animals.

Measurements of plasma insulin and glucose were not obtained in this experiment, however the drug treatment regimes applied were reproduced exactly from the literature reports of experiments that demonstrated clear improvements in the insulin sensitivity of the Zucker rats (**Wilbraham et al.**, 1994 and **Ozcelikay et al.**, 1994).

Thirty female lean and thirty female obese AP strain Zucker rats, approximately four months old, were used in this study. The animals were caged in pairs. In each of the lean and obese animal groups, five pairs were dosed with ZD2079, five pairs with vandyl sulphate and five pairs were given no drug treatment and acted as a control group.

Vanadyl sulphate (Sigma) was dosed to the animals in the drinking water. The agent was present in the drinking water at a concentration of 1 mg ml^{-1} and the water was available to the animals *ad lib*. This dosing regime was identical to that used by **Ozcelikay et al.**, (1994) whose experiment was outlined in the introduction section.

ZD2079 (Zeneca) was dosed at $50 \text{ mg kg}^{-1} \text{ day}^{-1}$ to the rats and was mixed into the ground rat diet provided. Fluctuations in the weights of the animals on test and their food intake were measured and the amount of drug placed in the diet corrected to produce the final dose of 50 mg kg^{-1} per day as described above.

The body weights, food and water intakes of the animals were measured on a regular basis prior to drug treatment to establish the normal basal values for each parameter. Once these basal measurements had been obtained, drug treatment was commenced (the body weights, food and water intakes of the animals were continually recorded during the drug dosing period).

The vanadyl sulphate dosing was commenced four weeks prior to the ZD2079 dosing. This was done to ensure that the three month vanadyl sulphate and the two month ZD2079 dosing periods ended simultaneously and so the vascular reactivities of the animals could be assessed at the same time.

Results

No significant differences were found between the groups of obese rats prior to the starting of drug treatments. The starting weights of the rats were $336.7 \pm 9.2 \text{ g}$ in the control group, $325.3 \pm 6.6 \text{ g}$ in the ZD2079 group and $345.5 \pm 4.9 \text{ g}$ in the vanadyl sulphate group (all groups contained ten rats).

The two drug treatments had significant effects on weight gain in the obese AP Zucker rats. At the end of the drug dosing period (i.e., at the time of pithing) the rats in the control group weighed $529.2 \pm 21.0 \text{ g}$, the rats in the ZD2079 group weighed $418.2 \pm 12.3 \text{ g}$ and the rats in the vanadyl sulphate group weighed $431.9 \pm 11.3 \text{ g}$. The difference between the control animal weights and the ZD2079 treated animal weights was statistically significant

($P < 0.001$ by unpaired t-test) as was the difference between the control and vanadyl sulphate treated rats ($P < 0.001$ by unpaired t-test).

The ZD2079 treatment reduced the weight gain of the obese animals. The treated animals gained weight at the rate of 0.9 ± 0.4 g day⁻¹ while, over the same period, the control animals gained 1.8 ± 0.3 g day⁻¹. The vanadyl sulphate treatment produced a similar effect, the treated animals gained 0.8 ± 0.4 g day⁻¹ while the control animals gained 2.0 ± 0.3 g day⁻¹ over the same time period.

The ZD2079 treatment, after producing an initial fall in food intake, subsequently produced a slight increase in the daily food intakes of the obese AP Zucker rats. Each of the treated animals consumed an average of 27.3 ± 1.1 g day⁻¹ of diet during the treatment period while the animals on control diet consumed 24.3 ± 0.6 g day⁻¹ during the same period. The water intake mirrored the food intake in the ZD2079 treated animals in that an initial fall in intake was followed by an overall increase. After the initial fall in water intake each ZD2079 treated animal consumed 32.0 ± 1.5 ml day⁻¹ while the each control treated animal consumed 25.3 ± 1.1 ml day⁻¹.

Vanadyl sulphate treatment produced large falls in both food and water intakes in the obese AP Zucker rats. During the drug dosing period the vanadyl sulphate treated animals each consumed 17.6 ± 0.3 g day⁻¹ of diet while the control group of rats each consumed 24.5 ± 0.5 g day⁻¹. The water intake of the vanadyl sulphate treated animals also was reduced compared to the control group, the treated animals each consumed 12.2 ± 0.6 ml day⁻¹ while the control animals each consumed 25.5 ± 1.0 ml day⁻¹.

No significant differences were detected between the groups of lean rats prior to the start of the two drug treatments. The starting weights of the rats were 183.6 ± 3.6 g in the control group, 171.0 ± 7.3 g in the ZD2079 group and 179.9 ± 4.3 g in the vanadyl sulphate group (all groups contained ten rats).

At the end of the dosing periods (i.e., at the time of pithing) the lean rats of the control group weighed 232.9 ± 4.8 g, the ZD2079 treated rats weighed 217.7 ± 9.2 g and the vanadyl sulphate treated animals weighed 206.4 ± 4.1 g.

The two drug treatments had less effect on weight gain in lean than in obese animals. The rats treated with ZD2079 gained weight at the same rate as the control animals (0.5 ± 0.1 g day⁻¹). The vanadyl sulphate treated rats gained an average of 0.3 ± 0.2 g day⁻¹ while the control animals gained 0.5 ± 0.1 g day⁻¹ over the same period.

The two drug treatments were much less effective in altering food and water intakes in the lean rats compared to their effects in the obese animals. The vanadyl sulphate treated animals each consumed 14.6 ± 0.3 g day⁻¹ of diet compared to the intake of 16.7 ± 0.1 g day⁻¹ in the control group over the same time period. The water intakes of the vanadyl sulphate treated animals was 13.7 ± 0.6 ml day⁻¹ compared with 21.1 ± 0.5 ml day⁻¹ in the control group.

The ZD2079 treatment again produced an initial transient decrease in food and water intakes in the lean Zucker rats followed by a sustained increase. Treated animals each consumed 18.1 ± 0.2 g day⁻¹ of diet compared to an intake of 16.7 ± 0.1 g day⁻¹ over the same period in the control animals. Treated animals each drank 22.1 ± 0.7 ml day⁻¹ of water compared with an intake of 21.2 ± 0.5 ml day⁻¹ in the control group.

Significant differences were found between the blood pressure responses to phenylephrine doses in the control lean and obese rats. These results are outlined in **Table 3.6.1** below.

Table 3.6.1 : Lean and Obese AP Zucker rat Blood Pressure Responses to i.v. Phenylephrine

<u>Phenylephrine</u> ($\mu\text{g kg}^{-1}$)	<u>Increase in dBP (mmHg)</u>		<u>Significance*</u>
	<u>Obese Rats</u> (n=10)	<u>Lean Rats</u> (n=9)	
1	10 \pm 2.9	7 \pm 1.3	NS
3	21 \pm 5.6	16 \pm 4.4	NS
10	72 \pm 12.9	56 \pm 8.4	NS
30	117 \pm 7.4	95 \pm 5.9	$P < 0.05$
100	143 \pm 7.0	125 \pm 5.4	$P < 0.01$

* by unpaired t-test

This data collection is also represented in graph format as **Figure 3.6.1**.

Figure 3.6.1 A Comparison of the Blood Pressure Responses to Phenylephrine of Untreated Lean and Obese AP Zucker Rats

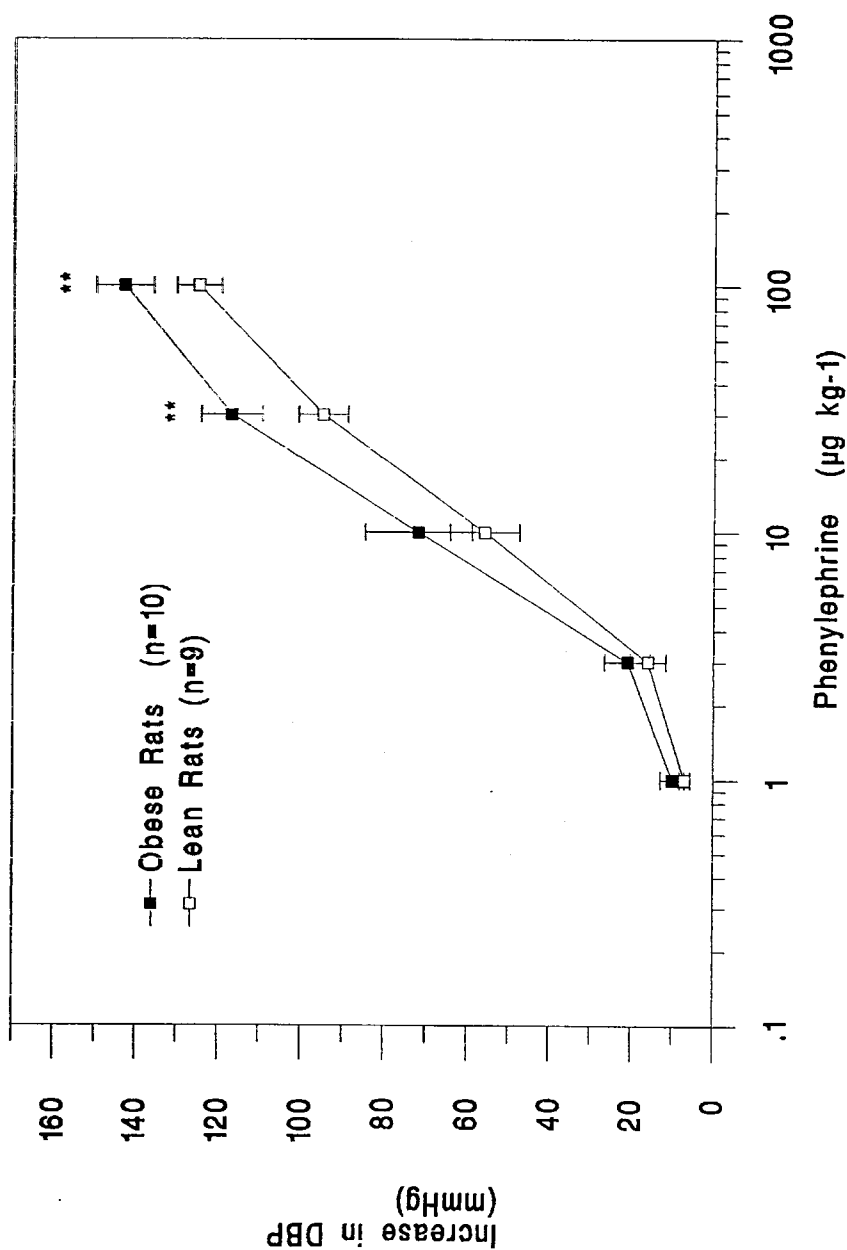


Figure 3.6.1 Groups of lean and obese female AP Zucker rats were fed a control diet for a period of four months then pithed. The increases in diastolic blood pressure (mmHg) due to i.v. phenylephrine in the obese (n=10) and lean (n=9) rats are illustrated. Significant differences were found between the two groups at the 30 and 100 $\mu\text{g kg}^{-1}$ dose (both $P < 0.01$ by unpaired t-test).

These results are very similar to those seen previously when both male and female lean and obese AP Zucker rat reactivities were compared in similar aged animals. Previous results have also demonstrated that pioglitazone was capable of reducing the increased reactivity observed in the obese animals.

(2) Drug Effects on Vascular Reactivity in the Obese Rats

The two drug treatments failed to produce any significant effects on the vascular reactivity of the treated obese rats.

Table 3.6.2 : The Effects of the Two Drug Treatments on the Vascular Reactivity of the Obese AP Zucker Rats.

<u>Phenylephrine</u> ($\mu\text{g kg}^{-1}$)	<u>Increase in dBP (mmHg)</u>		
	<u>Control Rats</u> (n=10)	<u>ZD2079 Treated</u> (n=9)	<u>Vanadyl Sulphate Treated</u> (n=10)
1	10 \pm 2.9	7 \pm 2.4	10 \pm 2.8
3	21 \pm 5.6	20 \pm 7.5	22 \pm 7.7
10	72 \pm 12.9	75 \pm 9.7	72 \pm 15.8
30	117 \pm 7.4	112 \pm 2.2	110 \pm 16.1
100	143 \pm 7.0	136 \pm 3.6	143 \pm 9.7

These results are expressed graphically as **Figure 3.6.2**.

(3) Drug Effects on the Vascular Reactivity of the Lean Rats.

Both drug treatments failed to produce any alterations in the vascular responses of the lean Zucker rats in this experiment.

Figure 3.6.2 The Effect of ZD2079 and Vanadyl Sulphate on the Blood Pressure Responses of Obese Female AP Zucker Rats to Phenylephrine

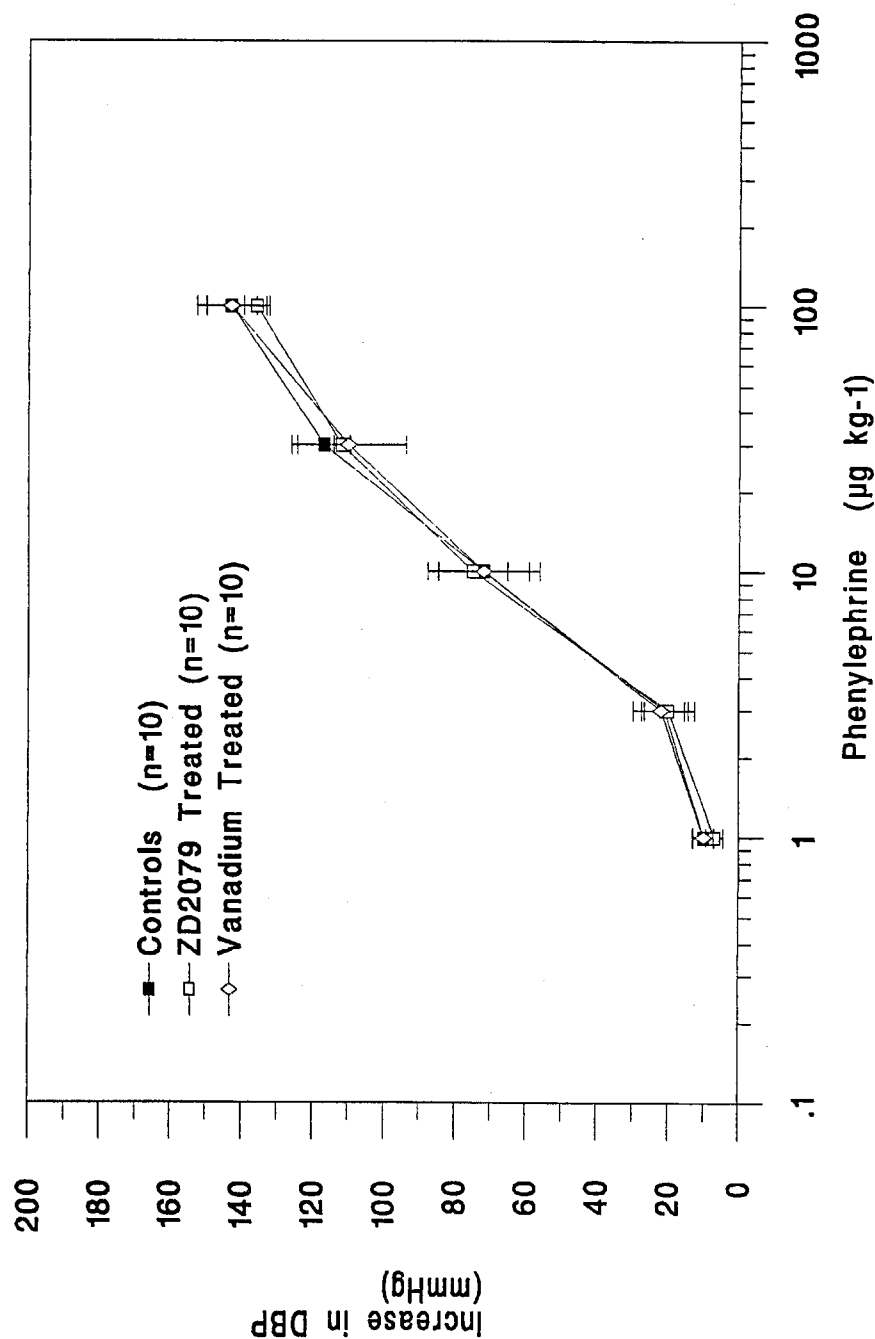


Figure 3.6.2 Groups of obese female AP Zucker rats were treated with ZD2079, vanadyl sulphate or vehicle as described elsewhere. At the end of the treatment period the rats were pithed. The increases in diastolic blood pressure (mmHg) due to i.v. phenylephrine in ZD2079 treated (n=10), vanadyl sulphate treated (n=10) and vehicle treated (n=10) rats are illustrated. no significant differences were found between the three groups.

Table 3.6.3 : The Effects of Drug Treatment on the Vascular Reactivity of the Lean AP

Zucker Rats.

<u>Phenylephrine</u> ($\mu\text{g kg}^{-1}$)	<u>Increase in dBP (mmHg)</u>		
	<u>Control Rats</u> (n=9)	<u>ZD2079 Treated</u> (n=9)	<u>Vanadyl Sulphate Treated</u> (n=8)
1	7 \pm 1.3	11 \pm 2.6	8 \pm 3.1
3	16 \pm 4.4	21 \pm 4.6	12 \pm 2.7
10	56 \pm 8.4	65 \pm 6.7	54 \pm 6.1
30	95 \pm 5.9	93 \pm 4.5	96 \pm 10.1
100	125 \pm 5.4	117 \pm 4.8	131 \pm 8.5

These results are expressed graphically as **Figure 3.6.3**.

Comments

In this experiment the vascular reactivity difference between the lean and obese AP Zucker rats was again observed (which implied that the vasculature of the obese animals tested was hyper-reactive). The two drug treatments produced no alteration of the vascular reactivity of either the lean or the obese animals tested despite significant alterations in the body weight and food and water intakes of the obese rats in particular.

Figure 3.6.3 The Effect of ZD2079 and Vanadyl Sulphate on the Blood Pressure Responses of Lean Female AP Zucker Rats to i.v. Phenylephrine

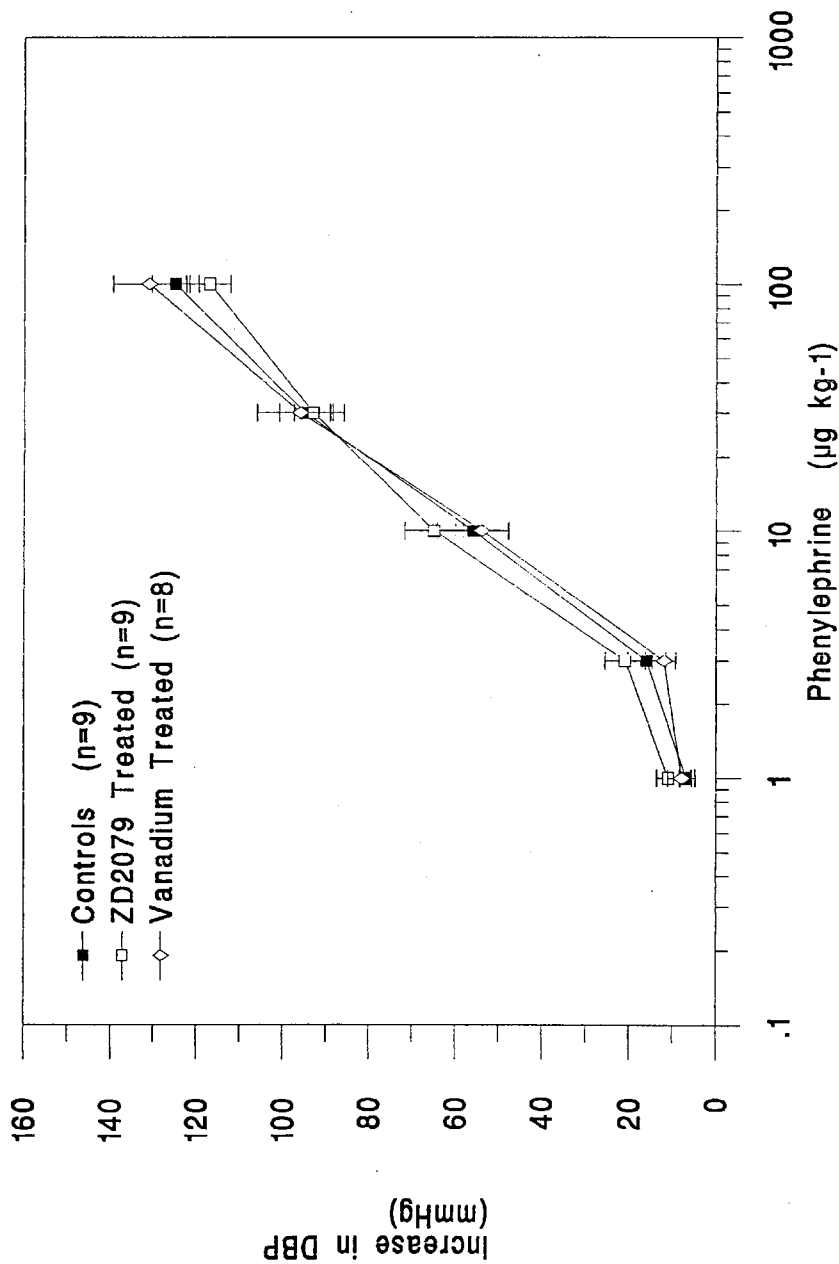


Figure 3.6.3 Groups of lean female AP Zucker rats were treated with ZD2079, vanadyl sulphate or vehicle as described elsewhere. At the end of the treatment period the rats were pithed. The increases in diastolic blood pressure (mmHg) due to i.v. phenylephrine in ZD2079 treated (n=9), vanadyl sulphate treated (n=8) and vehicle treated (n=9) rats are illustrated. no significant differences were found between the three groups.

Section Four

Discussion

In this section the results are discussed in the context of the original aims of the project (as set out in the introduction section). The aims of the project were to :

- (1) Determine if a functional difference existed between the vasculatures of animals with normal insulin sensitivity and those of animals with impaired insulin sensitivity.
- (2) Determine how insulin sensitising agents affect the vascular reactivities of insulin resistant and insulin sensitive animals.
- (3) Determine if any effects observed on the vascular reactivity of insulin resistant animals influence the blood pressure of the animals.

(1) Is Insulin Resistance Associated with an Increased Vascular Reactivity ?

As stated at the outset of the project, it was hoped to investigate both an experimentally induced (dietary) and a spontaneous (genetic) animal model of IR and to examine the vasculatures of each model with reference to a comparable animal with a normal insulin sensitivity.

In the experiment described in section 3.2, the high fat diet failed to induce an increase in the vascular reactivity of the AP Wistar rat. It must be stated that no measurements of insulin and glucose were made during this experiment so the insulin resistant state of the rats could not be demonstrated. However, as described in the results section, the 30 % fat diet used in this study had been shown to induce decreased insulin sensitivity over a similar treatment period in the AP Wistar rat in experiments carried out at Alderley Park.

Evidence from the literature on experiments where dietary manipulation has been used to bring about IR (and hence vascular reactivity and BP changes) indicates that rat strain, and indeed sub-strain, is an important variable. **Zein et al.**, (1990) described a study in which a high sucrose diet was fed to three different sub-strains of Wistar rat in an attempt to induce alterations in BP. It was found that the BP of Wistar-Kyoto strain rats was less responsive to dietary manipulation than other normotensive Wistar strains. **Preuss and Preuss** (1980) had previously published similar data, again using a high sucrose diet, demonstrating that

the American Wistar rat was less liable to dietary-induced hypertension than the Wistar-Kyoto strain rat.

Another potentially important experimental variable, which was not examined in the study carried out as part of this project, was the composition of the diet to which the 30% fat was added. It has been demonstrated that an increase in oat bran content of rat diet can reduce the increased BP induced by increased sucrose ingestion (**Zein et al.**, 1990). Zein et al., suggested that oat bran may have acted to prevent an increase in BP in this experiment by either increasing faecal water loss (lowering circulating blood volume) or by altering the rate of nutrient and water absorption from the intestinal lumen by decreasing the transit time of material through the intestine. It may have been the case that the normal ground diet used in the study carried out as part of this thesis had a sufficient roughage content to counter the effects of the added fat by the mechanisms outlined above.

The experiment described above indicated that fat feeding had little effect on the vascular reactivity of the AP Wistar rat. Further investigation of the vascular effects of fat feeding would involve the assessment of rat strain differences and of other components of the animal diet used. These experiments were beyond the scope of this study.

There is a large body of evidence in the literature that indicates that the obese Zucker rat is associated with marked IR (e.g., **Kurtz et al.**, 1989). A statistically significant difference between the vascular reactivities of the lean and obese AP Zucker rat was seen in all the experiments carried out as part of this project. The difference in reactivity between the lean and obese animals was present in both male and female Zucker rats. This difference was observed even in the presence of surgical stress and anaesthesia, which were a feature of some experiments carried out to establish a standard experimental methodology. This data supports the observations of **Zemel et al.**, (1992), among others, who describe the obese Zucker rat as hyper-reactive compared to the lean Zucker rat. Given that the obese Zucker rat is insulin resistant, when compared with the lean Zucker rat, the observation that the obese animal has an increased level of vascular reactivity compared to the lean animal suggests, at the very least, that there may be an association between IR and changes in vascular reactivity.

It was interesting to compare the basal reactivity levels of the two AP Zucker rat phenotypes with the other rats strains investigated (please refer to **Figure 4.1**). Various literature reports have described the obese Zucker rat as a model of vascular hyper-reactivity. Given the comparisons of the vascular reactivity of the obese Zucker rat with that of the AP Wistar rat and AP SH rat, it was difficult to describe the obese Zucker rat as an example of vascular hyper-reactivity in the experiments carried out for this project. Indeed, from the results presented in **figure 4.1**, it may be as valid to describe the lean AP Zucker rat as an example of vascular hypo-reactivity.

Despite the modest level of vascular reactivity observed in the obese animals compared to the other rat strains tested, it was still true to say that the obese rats were hyper-reactive when compared to their lean phenotype. As the obese animals were also insulin resistant in comparison to their lean counterparts, it was concluded that the lean and obese Zucker rat phenotypes offered a valid model in which to study the link between vascular reactivity and IR.

(2 and 3) Do agents that improve insulin sensitivity affect vascular reactivity and blood pressure ?

Given that a difference was found between the vasculatures of the obese and lean AP Zucker rat, more direct evidence was sought which would link the vascular difference with the difference in insulin sensitivity. Assuming that a known insulin sensitising agent would be effective in increasing the insulin sensitivity of the obese AP Zucker rat, a decrease in vascular reactivity due to the drug treatment would indicate that insulin sensitivity was linked to the vascular reactivity of the animal.

Figure 4.1 The Blood Pressure Responses of the Lean and Obese Zucker Rat, the AP Wistar Rat and the AP SH Rat to i.v. Phenylephrine

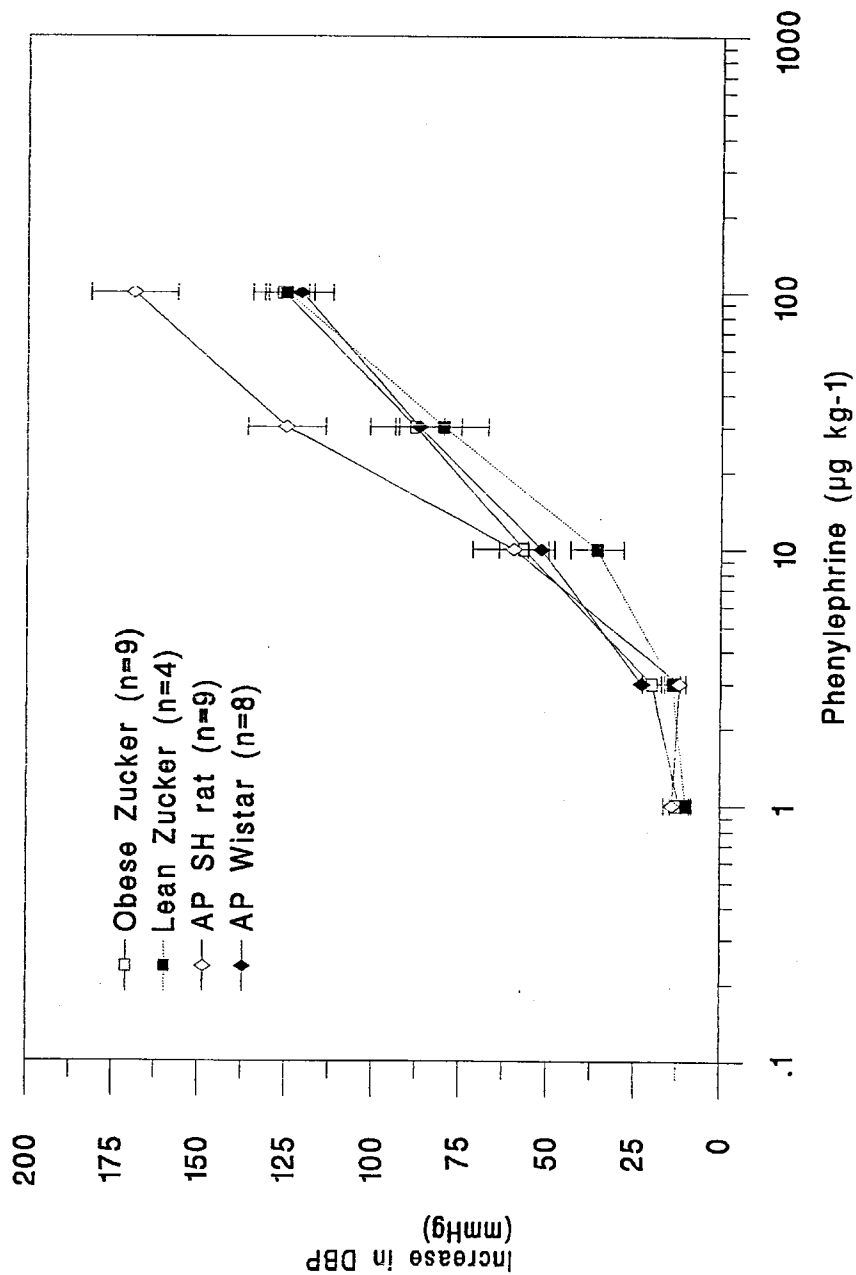


Figure 4.1 The data presented above represent the blood pressure responses of untreated, control groups of obese and lean AP Zucker rats, AP Wistar rats and AP SH rats from various experiments carried during the course of this project.

Pioglitazone has been shown to improve insulin sensitivity in a number of animal models (e.g., **Kemnitz et al.**, 1994 and **Ikeda et al.**, 1990) including the obese Zucker rat (**Doebber et al.**, 1993). In the experiments described in section 3.3, treatment with pioglitazone produced statistically significant falls in the reactivity of obese, but not of the lean Zucker rats. However, pioglitazone treatment produced this effect in both male and female rats. Pioglitazone produced no significant alterations in the vascular reactivity of either the AP Wistar rat or the AP SH rat. The AP Wistar rat has a normal insulin sensitivity while the insulin resistant status of the AP SH rat is controversial (as described in the introduction section). One implication that could be drawn from this data is that the pioglitazone treatment produced a decrease in the vascular reactivity of the insulin resistant obese Zucker rat due to the insulin sensitising properties of the agent.

It is possible that the lack of effect of pioglitazone treatment on vascular reactivity in the AP SH rat could have been due to the very high reactivity of the animal. It may have been the case that the reactivity of the SH rat was maximal and was not sensitive to alteration by an agent, such as pioglitazone, with moderate vasoactive properties as the reactivity was still maintained via other mechanisms. Conversely, the reactivity level of the lean AP Zucker rat may have been too low to be reduced further by drug treatment. That said, the AP Wistar rat demonstrated a similar basal vascular reactivity level to the obese AP Zucker rat. Even assuming that the vasculatures of the SH and lean Zucker rats were insensitive to drug alteration, if pioglitazone was acting via a mechanism other than one concerned with insulin sensitivity, it would have been expected that the agent would have produced a similar decrease in the vascular reactivity of the AP Wistar rat to that observed in the obese Zucker rat.

In the experiments described above, the insulin sensitising agent pioglitazone produced decreases in the vascular reactivity of the insulin resistant obese Zucker rat. This is further positive evidence to support the hypothesis that an insulin resistant state supports an increased vascular reactivity.

Work carried out using a radio-telemetry system within our group demonstrated that the obese AP Zucker rat was hypertensive when compared to the lean AP Zucker rat. Since

others were also involved with this work, the results of this work are presented in **Appendix 1**. Given that an insulin sensitising agent, pioglitazone, had been shown to decrease the vascular reactivity of the insulin-resistant obese rat, the effects of the agent on the blood pressure levels of the two AP Zucker rat phenotypes were of much interest. A pioglitazone induced BP fall in the obese AP Zucker rat but not in the lean rat would have indicated that the reactivity level of the obese animal was important in maintaining the elevated BP level (compared to the lean animal). The results of this experiment, which was carried out within our group, are presented in **Appendix 1**.

Pioglitazone produced small but significant decreases in the BP of both the lean and obese AP Zucker rat and also in the BP of the AP SH rat. Unlike the experiments measuring vascular reactivity, the data from the experiments measuring the effects of pioglitazone on BP suggest that the agent did not just have an effect in the insulin resistant obese AP Zucker rat. This result is in agreement with a report in the literature in which pioglitazone has been shown to be a hypotensive agent in both insulin resistant animals as well as in animal models in which a normal insulin sensitivity is present. **Zhang et al.**, (1994) demonstrated that pioglitazone treatment produced BP falls in both the insulin resistant Dahl salt-sensitive rat and in the one-kidney, one clip rat model (a model of renal hypertension with a normal insulin sensitivity).

A comparison of the pioglitazone induced BP falls produced in the three rat strains indicated that, on the fourth day of pioglitazone treatment, the basal BP of the telemetered rats fell, when compared to the fourth day of vehicle dosing, by an average of 7 mmHg in the AP SH rat (n=4), 7 mmHg in the lean AP Zucker rat (n=8) but a larger 19 mmHg in the obese AP Zucker rat (n=6). When expressing these BP falls as a percentage of the initial starting control BP level for each rat strain, it can be clearly seen that pioglitazone had a larger effect on BP in the obese AP Zucker rat (a fall of 15.4 %) than in the lean AP Zucker rat or the AP SH rat (falls of 6.9 % and 4.2 % respectively).

One implication that may be drawn from this data is that the larger BP fall seen in the pioglitazone treated obese AP Zucker rat was connected with the reduction in vascular reactivity induced by the same pioglitazone treatment, as demonstrated in the experiments on pithed animals described previously. Thus, these experiments may also demonstrate,

although indirectly, that the enhanced reactivity of the obese Zucker rat may help to support a hypertensive state.

A major criticism of the experiment using vanadyl sulphate and ZD2079 as insulin sensitising agents (and indeed of the other experiments carried out as part of this project) is that no measurements of insulin and glucose were made. Because of this, no measure of insulin sensitivity, and hence changes in insulin sensitivity, of the treated rats was obtained. That said, both the vanadyl sulphate and ZD2079 treatment regimes used in this experiment produced definite changes in the metabolism of the obese and lean AP Zucker rats, as evidenced by the changes in weight gain and food and water intake of the animals, in comparison with the control treated group of animals. In view of these changes, and the literature precedent with the two treatment regimes, it was assumed that a concurrent improvement in insulin-sensitivity had occurred in the animals. Despite these changes, however, the vascular reactivity level of both the obese and lean vanadyl sulphate and ZD2079 treated AP Zucker animals remained unaltered from that of the untreated, control, animals. Therefore, improvements in insulin sensitivity may not always result in a decreased vascular reactivity in insulin resistant animals.

The vanadyl sulphate treatment regime used in this experiment has been shown to decrease the reactivity of blood vessels from treated animals when reactivity was measured *in vitro* (Ozcelikay et al., 1994), so it was surprising that no decrease in vascular reactivity was observed in this experiment. Other literature reports, however, have demonstrated that vanadium containing compounds have the ability to enhance protein tyrosine phosphorylation (via an inhibition of tyrosine phosphatase) and cause a direct constriction of blood vessels (Laniyonu et al., 1994 and Di Salvo et al., 1993). One explanation for the apparent lack of activity of vanadyl sulphate treatment on vascular reactivity in this experiment may be that vanadyl sulphate induced two counteracting effects on the vasculature of the treated animals, a decrease in reactivity due to an improved insulin sensitivity and a simultaneous increase in reactivity due to an inhibition of tyrosine phosphatase.

Insulin itself has also been demonstrated to potentiate contractile responses in vascular tissue. **Henrion and Laher** (1994) demonstrated that insulin potentiated the contractions of rabbit facial arteries induced by noradrenaline. These authors demonstrated that insulin produced this potentiation via increases in activation of protein kinase C and tyrosine kinases. This observation leads to the possibility that, in the experiment using vanadyl sulphate and ZD2079 as insulin sensitising agents in the obese AP Zucker rat, increases in insulin sensitivity potentiated both a relaxant and a contractile effect of insulin causing no net change in the overall vascular reactivity of the obese AP Zucker rat.

The dual effect of vanadate on the vasculature, i.e., vanadium containing compounds being capable of causing both an increase and a decrease in vascular reactivity, is also reflected in the literature of studies investigating the effects of the vanadium containing compounds on BP. **Bhanot and McNeill** (1994) described a set of studies in which vanadyl sulphate treatment caused reductions in both plasma insulin and BP levels in SH rats but not in normotensive Wistar-Kyoto rats. **Bhanot et al.**, (1994) describe further studies in which treatment with vanadyl sulphate prevented the BP rise caused by fructose feeding in male Sprague-Dawley rats. Conversely, **Burszty and Mekler** (1993) reported that treatment with vanadate induced hypertension in male Sabra rats by increasing their salt-sensitivity. Interestingly, the authors also found that the vanadate treated rats had an increased vascular responsiveness to i.v. angiotensin II administration compared to rats that had not been treated with vanadate.

Many studies have demonstrated that tissues from insulin resistant animals, when examined *in vitro*, are hyper-reactive to constrictor agents when compared to tissues from animals with a normal insulin sensitivity (e.g., **Cox and Kikta**, 1992 and **Zemel et al.**, 1991). Possibly the most interesting feature of these studies is that they have mostly been carried out in the absence of any added insulin in the organ bath. Even allowing for the possibility that a residual amount of insulin may still have been present in the tissues studied, it is clear from these experiments that bathing concentrations of insulin are not vital in demonstrating differences between the vascular tissues of animals with differing insulin sensitivities. The main conclusion from these experiments is that the tissues from

insulin resistant animals and those from animals with a normal insulin sensitivity have different responses to constrictor agents regardless of local insulin concentrations (i.e., their *in vitro* responses are not different due to different sensitivity to an acute insulin mediated dilation effect, as no insulin is present).

A further conclusion that may be drawn from these experiments is that a chronic insulin resistant state produces an irreversible, or at least a long lasting, adaptive change to the peripheral vasculature causing it to be hyper-reactive to constrictor agents regardless of local insulin concentrations (perhaps similar to the effect of calcium-loading of vascular smooth muscle as seen in models of chronic hypertension). Such adaptive changes to insulin might explain the lack of effect of the vanadyl sulphate and ZD2079 treatments on the vascular responses of the obese AP Zucker rats. The obese AP Zucker animals used in the study were in a well established insulin resistant state prior to the treatment with the two insulin sensitising agents. Treatment with the two agents produced metabolic changes in the obese rats leading to an improved insulin sensitivity (as evidenced by their smaller weight gains compared to untreated control animals). If, however, the previously existing insulin resistant state had produced an irreversible adaptive change in the vasculature of the animals then an improved insulin sensitivity would not have fully restored the level of responsiveness of the vasculature to that present prior to the onset of the insulin resistant state.

However, if a permanent change is induced in the vasculature of animals due to chronic IR the lack of effect of the vanadyl sulphate and ZD2079 treatment in the obese AP Zucker rat in the experiments carried out for this project can be explained. It is difficult to explain the fact that pioglitazone was able to produce decreases in the reactivity of the obese AP Zucker rat vasculature. Previously it was assumed that the insulin sensitising properties of pioglitazone were behind the decrease in vascular reactivity of the obese AP Zucker rat i.e., that pioglitazone decreased the vascular reactivity of the insulin resistant rat by potentiating the relaxant properties of insulin on the vasculature. The failure of both vanadyl sulphate and ZD2079 treatment to alter the vascular reactivity of the obese AP Zucker rat would

suggest that such a reduction in IR does not invariably lead to a decrease in vascular reactivity.

This would imply that pioglitazone has actions, unrelated to its insulin sensitising properties, which act to decrease vascular reactivity and hence BP. Pioglitazone has been shown to have effects on L-type calcium channels in aortic smooth muscle cells (Zhang et al., 1994) and to inhibit calcium uptake by vascular smooth muscle induced by norepinephrine, vasopressin and potassium chloride (Buchanan et al., 1995). These actions would result in inhibition of vascular smooth muscle contractility and hence would decrease vascular reactivity even in animal models with a normal insulin sensitivity.

The experiments carried out as part of this project have demonstrated that pioglitazone has a modest, but significant, hypotensive effect in the AP SH rat and the lean AP Zucker rat, two rat strains with, respectively, a normal insulin sensitivity and, in the case of the AP SH rat, at best a modest impairment of insulin sensitivity. These data would support the view that the observed activity of pioglitazone was unrelated to the insulin resistant state of the test animal. Despite this, however, in the experiments carried out in pithed animals, pioglitazone only altered the vascular reactivity of obese AP Zucker rats. The conclusion drawn from these experiments is either that pioglitazone was acting on vascular reactivity by altering the insulin resistant state of the obese animal or that the obese rat possessed a secondary, independent, defect in its vasculature which was both sensitive to pioglitazone treatment and not shared by the lean AP Zucker, the AP Wistar or the AP SH rat.

Future Work

The experiments carried out as part of this project, as noted in the discussion above, indicated that IR has a strong link with an increased vascular reactivity but that this link may not be clear cut. Two main problems were found in the interpretation of the data from the experiments. The first of these problems was that no measurements of insulin sensitivity were obtained from any of the experiments. Known insulin sensitising agents were used during these experiments but their alterations in the reactivity of insulin resistant vasculatures could not be directly linked to the degree of change in insulin sensitivity. It

would be desirable to measure the change in insulin sensitivity induced by the pioglitazone treatment used in the experiments described above and link them with the vascular changes observed.

The second problem encountered during these experiments was that the insulin sensitising agents used had other actions, on vascular smooth muscle in particular, which made drug induced vascular alterations difficult to relate purely to changes in insulin sensitivity. Future experiments should concentrate on attempting to demonstrate the effect of a pure insulin sensitising treatment on the reactivity of the insulin resistant vasculature. One possible non-chemical treatment would be dieting. **Ikeda et al.**, (1996) demonstrated that weight loss in obese hypertensive patients produced concurrent falls in IR, as measured by euglycaemic clamp studies, and systemic BP. This clinical observation supported earlier work such as that reported by **Rocchini et al.**, in 1987. These authors demonstrated that BP falls induced by weight loss and exercise regimes in obese adolescents were associated with falls in plasma insulin (indicating that an improvement in insulin-sensitivity had been induced).

An experiment in the obese and lean Zucker rat could be carried out in which a dietary or exercise regime were used as an insulin sensitising treatment. Measurements of insulin sensitivity would be taken both prior to the imposition of the treatment and after the treatment so a quantitative measure of the improvement in insulin sensitivity could be obtained. The animals could be implanted with a radio-telemetry BP recording unit during the course of the experiment so that any alterations in BP could be recorded. At the end of the treatment period the animals could be pithed and the reactivity of their vasculature assessed and compared to a suitable control group. This experiment would be free of the problems of drug side-effects found during this project and also provide a quantitative measure of alterations in insulin sensitivity which could be related to the degree of vascular changes found.

Summary

I will now return to the questions posed at the start of the project (and restated at the beginning of this section) and briefly put forward the key observations that go towards answering them.

(1) The physiology / pathology of the insulin resistant vasculature.

Does a functional difference exist between the vasculatures of animals with normal insulin sensitivity and those of animals with impaired insulin sensitivity?

(2) The effects of insulin sensitising agents.

If a difference can be observed, how do insulin sensitising agents affect the vasculatures of insulin resistant and insulin sensitive animals?

In terms of vascular reactivity, the data discussed above can be divided into two categories, that which supports the hypothesis that IR is related to peripheral vascular changes and that evidence which opposes the hypothesis. Two main pieces of evidence indicate that IR is linked to an increase in vascular reactivity. These pieces of evidence are:

- (a) the insulin resistant obese AP Zucker rat demonstrates an increased vascular reactivity over that of the insulin sensitive lean AP Zucker rat
- (b) the insulin sensitising agent pioglitazone reduces the vascular reactivity of the obese AP Zucker rat but not that of more insulin sensitive animals (the lean AP Zucker, the AP Wistar and the AP SH rat).

The main piece of evidence supporting the contrary stance, that IR is not necessarily directly linked to an increase in vascular reactivity, was produced from the experiment where two insulin sensitising agents, vanadyl sulphate and ZD2079, failed to alter the level of vascular reactivity of the obese AP Zucker rat.

(3) *The influence of vascular reactivity on blood pressure.*

How do any effects observed on the vascular reactivity of insulin resistant animals influence the BP levels of the animals?

The BP levels of the obese and lean AP Zucker rats, as measured by radio-telemetry, mirrored the relative vascular reactivities of the two phenotypes as measured using the pithed animal methodology in that the hyper-reactive obese rat was hypertensive when compared to the less reactive lean rat. Some slightly more direct evidence was produced from this project which indicated that a reduction in the vascular reactivity of the obese AP Zucker rat, produced by pioglitazone treatment, was associated with an exaggerated BP fall when compared to the BP falls induced in other rat strains whose vascular reactivity was unaffected by pioglitazone treatment.

Appendix One

The Blood Pressure Difference Between Lean and Obese AP Zucker Rats.

Groups of male lean and obese AP Zucker rats were implanted with radio-telemetry BP recording equipment as part of an experiment carried out within our work group. For a full explanation of the radio-telemetry system please refer to section 2.3.3. The rats tested were observing a normal light dependent activity pattern. The animals were housed in a room that provided a 12 hour light period (6:00 AM to 6:00 PM) during which the animals were dormant and a 12 hour dark period (6:00 PM to 6:00 AM) during which the animals were active. The BP levels of the animals were directly related to their activity levels and so their BP levels also followed the twelve hour light and dark cycle. It was found that a significant BP difference existed between the lean and obese rats during both the light and dark periods of the light cycle ($P < 0.001$ by unpaired t-test in both periods). These results are presented in **Figure A.1**.

The Effect of Pioglitazone Dosing on the Blood Pressure of Lean and Obese Zucker Rats.

As part of a larger experiment carried out in our work group, pioglitazone was dosed to small groups of male lean and obese AP Zucker rats which had previously had BP recording radio-telemetry equipment implanted. At the time of the experiment the rats were six months old. In the experiment polysorbate vehicle was dosed p.o. once daily to the test animals for a period of four days. Following this control period, pioglitazone was dosed at a dose of 30 mg kg⁻¹ again once daily p.o.

The BP effects of this dosing are presented as **Figure A.2** (daytime means) and **figure A.3** (night time means).

The main conclusion drawn from this experiment was that pioglitazone produced a fall in both daytime and night-time BP readings in both the lean and obese AP Zucker rat. It was also obvious that pioglitazone produced a larger hypotensive effect in the obese animal (i.e., produced a differential effect in the two Zucker phenotypes).

Figure A.1 The Difference Between the Mean Blood Pressures of the Lean and Obese AP Zucker Rat as Measured by Radio-telemetry

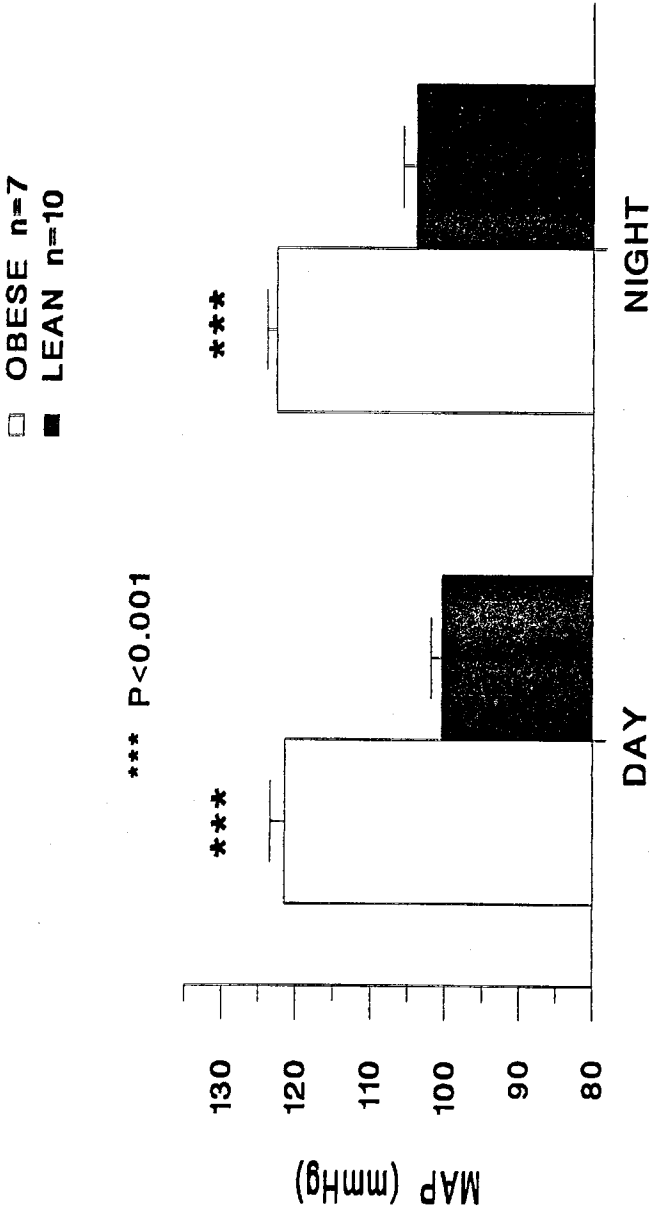


Figure A.1 Groups of male lean and obese AP Zucker rats were cannulated with Data Sciences blood pressure measuring telemetry units. The animals were allowed a week to recover from surgery. Twelve hour means (split into day time and night time) for the mean blood pressure of both the lean and obese groups are illustrated. A significant difference was found between the blood pressures of the obese and lean rats during both the day and night time periods (both $P<0.001$ by unpaired t-test).

Figure A.2 The Effect of Pioglitazone at 30 mg kg⁻¹ p.o. day⁻¹ on the Day Time Mean Blood Pressure of Lean and Obese AP Zucker Rats

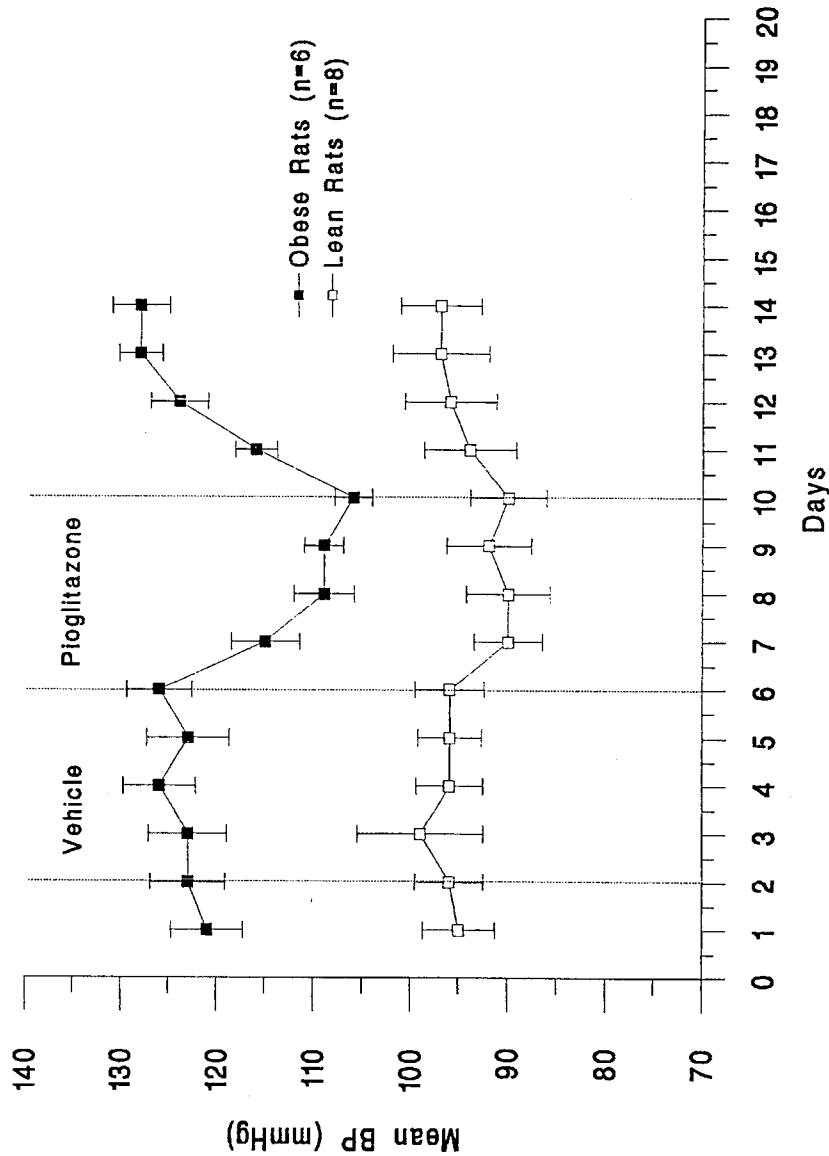


Figure A.2 Groups of lean and obese male AP Zucker rats, implanted with a radio-telemetry blood pressure measuring unit, were dosed with polysorbate for four days. Following this control period the rats were treated with 30 mg kg⁻¹ pioglitazone p.o. once daily for a period of four days. The day time mean blood pressure values are illustrated. On the fourth day of pioglitazone treatment a significant fall in mean blood pressure was observed in both the lean and obese rats (both $P<0.001$ by paired t-test) when compared to the values after four days of vehicle treatment.

Figure A.3 The Effect of Pioglitazone at 30 mg kg⁻¹ p.o. day-1 on the Night Time Mean Blood Pressure of Lean and Obese AP Zucker Rats

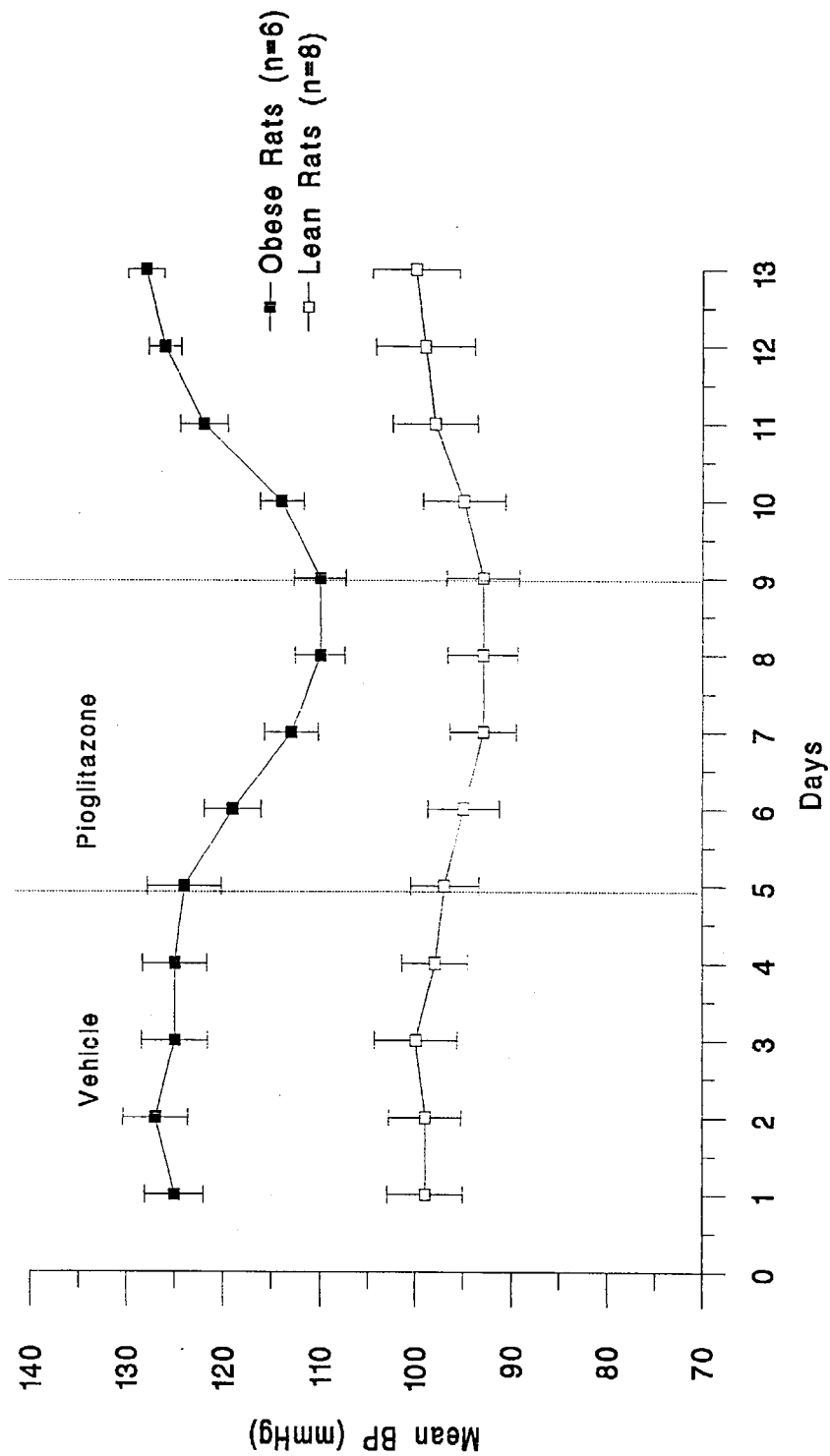


Figure A.3 Groups of lean and obese male AP Zucker rats, implanted with a radio-telemetry blood pressure measuring unit, were dosed with polysorbate for four days. Following this control period the rats were treated with 30 mg kg⁻¹ pioglitazone p.o. once daily for a period of four days. The night time mean blood pressure values are illustrated. On the fourth day of pioglitazone treatment a significant fall in mean blood pressure was observed in both the lean and obese rats (both $P < 0.001$ by paired t-test) when compared to the values after four days of vehicle treatment.

The pioglitazone dosing regime used in this experiment produced a haemodynamic change in the Zucker rat (the degree of which appeared to be different in the lean and obese animals) without demonstrating any adverse side effects. It was decided to use a similar regime in all the experiments involving the agent carried out subsequently.

Section Five

References

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